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TABLE OF CONTENTS

A Proposed Mechanism of Extracellular Regulation of Muscle Composition—

ROBERT E. COOKE, *Assistant Professor of Pediatrics and Physiology*, and WILLIAM E. SEGAR, *Instructor in Pediatrics, Yale University School of Medicine* 83

Rapid Micro Method for the Turbidimetric Determination of Serum Protein—

I. A. PARFENTJEV, *Research Associate in Microbiology, Yale University School of Medicine*, and MARIE-LOUISE JOHNSON, *Candidate in Microbiology, Yale University Graduate School* 94

Circulatory Changes in Pulmonary Arterio-Venous Fistula—

FRANK D. GRAY, JR., *Assistant Professor of Medicine, Yale University School of Medicine*, PAUL R. LURIE, *Assistant Professor of Pediatrics, University of Indiana School of Medicine, Indianapolis, Indiana*, and RUTH WHITTEMORE, *Associate Clinical Professor of Pediatrics, Yale University School of Medicine* 107

The Prerequisites of Good Teaching—

ERNEST SACHS, *Research Associate in Physiology, Lecturer in Surgery (Neurology), Yale University School of Medicine* .. 115

The Partial Purification of the Lethal Toxin of *Clostridium Histolyticum*—

HARRY E. BOWEN, *Chief Bacteriologist, Division of Biologic Laboratories, Department of Public Health, 375 South Street, Forest Hills, Boston 30, Massachusetts* 124

A Comparison of the Lethal and Hemolytic Toxins of *Clostridium Histolyticum*—

HARRY E. BOWEN, *Chief Bacteriologist, Division of Biologic Laboratories, Department of Public Health, 375 South Street, Forest Hills, Boston 30, Massachusetts* 131

Serum Vitamin A in Normal and A-deficient Monkeys (*Macaca Mulatta*)—

B. LIONEL TRUSCOTT, *Assistant Professor of Anatomy, University of North Carolina School of Medicine, Chapel Hill, North Carolina,* and GERTRUDE VANWAGENEN, *Research Associate in Obstetrics and Gynecology, Yale University School of Medicine* 139

A Jacobean View of Old Age—

LEONELL C. STRONG, *Research Associate in Anatomy, Yale University School of Medicine* 147

William Thomas Salter (1901-1952) 149

Events 151

Book Reviews 153

Yale Alumni in Medicine (Alumni edition only) i

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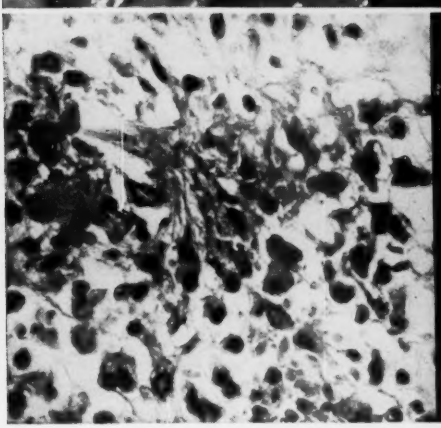

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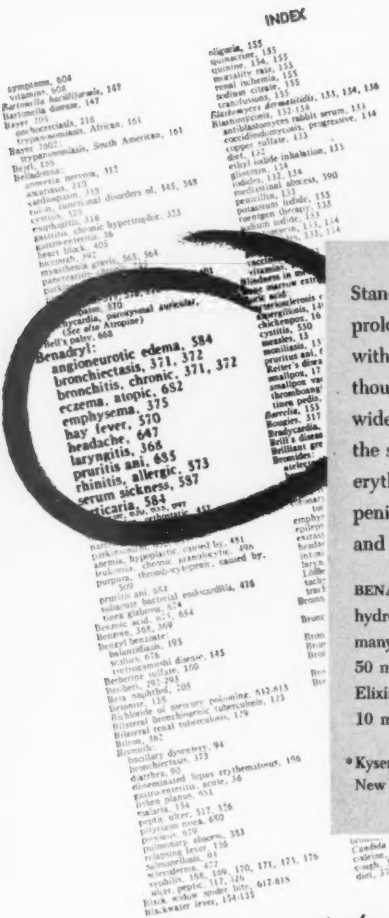

INDEX*
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A PROPOSED MECHANISM OF EXTRACELLULAR REGULATION OF MUSCLE COMPOSITION*

ROBERT E. COOKE** AND WILLIAM E. SEGAR

The changes in muscle composition which occur in disturbances of extracellular electrolyte composition were systematically described by Darrow, *et al.*[8,11] and confirmed by others.[7,15] However, the mechanism responsible for these changes has not been satisfactorily elucidated. It is the purpose of this paper to describe briefly the composition of muscle in the major disturbances of acid-base balance and to present a general theory of the mechanism of electrolyte transfer between cell and extracellular fluid which could account for these findings. It is important to note that relatively little of the data to be discussed subsequently was obtained by the authors and their contribution is simply the interpretation of these data.

Muscle electrolyte composition is usually expressed in terms of the fat-free solids (FFS) rather than as concentration per liter of intracellular or extracellular fluid. Since the quantity of muscle solid per kilogram of animal is relatively constant and not affected by changes in extracellular volume, analysis of muscle expressed in these terms permits valid comparison of data obtained in various disturbances. The results of acute experiments are not given since time must elapse before marked alterations in muscle composition occur. The analyses presented are those of skeletal muscle of rats only. Analysis of human muscle obtained by biopsy suggests that changes in cell composition in man are similar.[10]

Metabolic alkalosis of the extracellular fluid produced either by administration of sodium bicarbonate or loss of chloride in excess of sodium leads to characteristic changes in muscle composition (Table 1).[7,8] The potassium content is low, averaging 38mM/100g.FFS and the intracellular sodium content is elevated, 7.7mM/100g.FFS. In addition, there is probably a rise in the concentration of undetermined anion (possibly lactate) in the serum as calculated from the difference between the concentrations of sodium and the sum of the concentrations of chloride and bicarbonate even when the quantity of undetermined anion is corrected for the change in base binding capacity of blood proteins with change in pH.

Metabolic acidosis of the extracellular fluid produced either by administration of HCl or NH_4Cl or loss of sodium in excess of chloride leads to

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*From the Departments of Pediatrics and Physiology, Yale University School of Medicine. A portion of the studies described in this review was aided by research grants from the James Hudson Brown Memorial Fund of the Yale University School of Medicine and from the United States Public Health Service.

** John and Mary R. Markle Scholar in Medical Science.

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TABLE 1

BODY COMPOSITION IN DISTURBANCES OF ACID-BASE BALANCE

		Serum							*Muscle per 100 g. FFS*					
Group	*References*	Na mM/L	K mM/L	Cl mM/L	HCO_3 mM/L	*"Lactate"* ..	pH	pCO_2 mm.Hg.	H_2O g.	Cl mM	Na mM	K mM	P mM	Na_i* mM
Normals	8, 10, 7, 17, 18	141—146	4.1—5.4	99—102	21—25	..	7.35	45	334—346	5.1—7.2	9.4—10.6	46.1—48.9	32.0—34.0	3.1
Metabolic alk.	7	151	3.4	87	35	↕	7.44	53	314	3.9	11.9	37.8	30.4	7.7
Metabolic acid.	7	142	6.7	94	20	↕	7.28	44	337	4.5	6.1	49.8	33.2	1.6
Resp. acid.	5	143	4.8	85	41	↕	7.25	100	333	5.3	8.8	48.0	33.0	2.2
Resp. alk. (altitude)	10	143	5.6	124	↕	↕	↕	↕	342	9.1	12.0	45.0	32.5	3.5
K. def. alk.	6	145	2.7	93.0	30	?	7.50	38	334	5.7	18.5	34.1	34.2	11.9
Acute K excess (Group 8)	14	141	12.4	115	....	?	...	...	359	9.7	8.3	53.1	32.0	—2.3**

* Na_i—calculated as difference between total muscle sodium and extracellular sodium of muscle; concentration of extracellular sodium calculated from $[Na]_e = \frac{[Na]\text{serum}}{(H_2O)\text{serum}} \times .95$.

Volume of extracellular phase of muscle calculated from muscle chloride, assuming 1 mM of Cl/100 g. FFS is intracellular. Therefore, extracellular water of muscle $= \frac{(Cl)_m - 1}{\frac{[Cl]\ \text{serum}}{(H_2O)\,\text{serum} \times .95}}$

** No significance in acute experiments since chloride probably enters cell in potassium poisoning.

changes in muscle composition opposite to those described in metabolic alkalosis[7, 11] (Table 1). The potassium content of muscle is elevated, 50mM/100g.FFS, and the intracellular sodium is low, 1.6mM/100g.FFS. There is probably a fall in concentration of undetermined anion in the serum.

Recently, muscle composition has been determined in rats made acidotic by exposure to an atmosphere containing 10-15 per cent carbon dioxide for 2-3 weeks[5] (Table 1). The average potassium content is 48mM/100g.FFS, approximating the upper limit of normal. The intracellular sodium is 2.2mM/100g.FFS, somewhat lower than normal. In addition, the undetermined anion concentration of serum is probably lowered even when corrected for pH change. These changes are very different from those obtained in metabolic alkalosis even though the serum bicarbonate concentration is elevated and the serum chloride concentration is lowered in both of these conditions.

Exposure to low atmospheric pressure probably produces chronic respiratory alkalosis by inducing overbreathing.[8] Analysis of rats exposed to such conditions revealed the following composition of muscle: potassium content, 45mM/100g.FFS, intracellular sodium, 3.5mM/100g.FFS[10] (Table 1). The potassium content is somewhat lower than normal and the sodium higher than normal. Furthermore, the serum lactate concentration has been shown to be elevated in respiratory alkalosis, and this elevation is not due simply to the activity of breathing since serum lactate fell to normal when 5 per cent CO_2 was substituted for room air.[8] These changes are different from those obtained in metabolic acidosis even though the extracellular bicarbonate concentration is depressed and the chloride concentration elevated in both conditions.

As can be seen from the four examples above, intracellular sodium does not universally vary directly with extracellular bicarbonate concentration or inversely with extracellular chloride concentration as previously considered[11] but rather with extracellular pH[3] (Fig. 1). Likewise, intracellular potassium varies inversely with extracellular pH. It is only in metabolic alkalosis and acidosis that cell composition varies with extracellular bicarbonate concentration since in these states $(pH)_e$* and $(HCO_3)_e$ vary in the same direction.

Excessive loss of potassium from the body usually leads to a rise in extracellular bicarbonate concentration. The muscle potassium is low in this condition (34mM/100g.FFS), and the intracellular sodium of muscle is elevated (11.9mM/100g.FFS).[6, 11] The increment of sodium amounts to approximately two-thirds of the decrement of potassium.[11, 17, 18] The difference (one-third) is considered to be hydrogen ion transferred from extra-

* Subscripts "e" and "i" refer to extracellular and intracellular phases respectively.

cellular fluid to cell to maintain electrical neutrality. The repair of alkalosis associated with potassium deficiency by means of potassium chloride administration does not require specific renal correction of the alkalosis and involves transfer of hydrogen ion from cell to extracellular fluid in exchange for potassium.[8]

Excess of potassium in the extracellular fluid can be maintained for only a short time in the presence of normal renal function, so that the converse of the above experiment cannot be performed readily. In acute experiments loads of potassium chloride produce a fall in extracellular bicarbonate con-

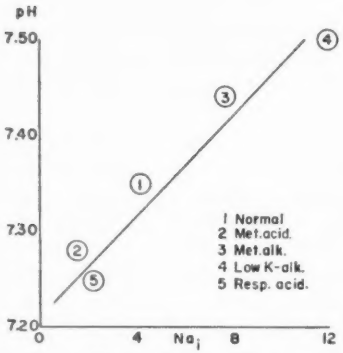

FIG. 1. Relationship of pH of extracellular fluid to intracellular sodium content of muscle expressed as mM/100 g. FFS.

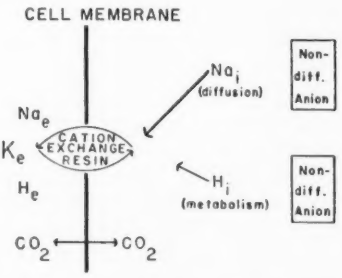

FIG. 2. Proposed mechanism of regulation of cell composition showing competition between intracellular sodium and hydrogen for exchange with extracellular potassium.

centration as calculated from the difference between extracellular sodium and chloride concentrations.[14] In these experiments muscle potassium is elevated and sodium depressed (53mM/100g.FFS and —2.3mM/100g. FFS, respectively).

The high potassium, low sodium content of cells has been attributed to a specific cation exchange system which permits transfer of sodium outward and potassium into the cell.[19] The data presented above suggest that this reaction may be concerned with transport out of the cell of hydrogen ion as well as sodium. This transport is assumed to be an equilibrium reaction to which the Law of Mass Action is applicable. Therefore, a change in concentration of the reactants on one side of the reaction leads to an alteration in the equilibrium point with a change in concentration of the reactants on the other side.

With these considerations it is possible to formulate a theory of electrolyte transfer at the cell membrane which could explain the alterations in muscle composition observed with changes in extracellular electrolyte com-

position. It may be postulated that intracellular sodium (entering the cell by diffusion) and intracellular hydrogen (resulting from cell metabolism) compete with each other at the cell membrane for a cation exchange system. This system, which perhaps has properties like an exchange resin, transfers intracellular sodium or hydrogen outward and extracellular potassium into the cell (Fig. 2). The quantity of sodium or hydrogen which is transported outward in exchange for potassium transported inward depends upon the concentrations of these materials on either side of the reaction. The shift in equilibrium point in this reaction is thought to account for changes in cell composition. It is noteworthy that, in terms of this theory, the loss of potassium from the cell or the movement of sodium into the cell is of little importance as regards cell composition. Since both of these processes take place by passive diffusion, they are dependent simply upon the difference in concentration between cell and extracellular fluid. Passive diffusion, therefore is essentially constant despite large changes in active exchange since the differences in concentration between cell and extracellular fluid are altered little by large percentage changes in extracellular potassium or intracellular sodium concentration. For example, a *50 per cent* fall in extracellular potassium concentration increases the cell to extracellular fluid gradient from 155mM/L (160 (cell)—5 (Excell.)) to 157.7mM/L (160 (cell)—2.5 (Excell.)), a *1.6 per cent* increase. Likewise, intracellular hydrogen ion concentration is considerably higher than extracellular hydrogen ion concentration (pH 6.1 and 7.4, respectively), and is probably maintained at a fairly uniform level because of the constancy of cell metabolism. Thus, the equilibrium point of the reaction, $(K)_e$ for (H)i is more likely to be influenced by changes in extracellular hydrogen ion concentration but may be influenced by alterations of intracellular hydrogen ion concentration resulting from changes in production of (H)i such as those which occur in febrile illnesses and during exercise. It is probable also that hydrogen ion leaves the cell not only by exchanging with potassium but also as H_2CO_3 or H— organic anion. The form in which potassium leaves the cell is considered to be free cation diffusing out as sodium diffuses in. In addition, potassium that enters the cell in exchange for intracellular hydrogen probably leaves the cell with diffusible anion which has resulted from cell metabolism. Otherwise, potassium would accumulate without limit within the cell. Since diffusible anion (probably lactate) is produced at the same rate as hydrogen ion by cell metabolism, the rate of diffusion out of the cell of this anion with accompanying potassium ion is proportional to (H)i for $(K)_e$ exchange. Therefore, at times when $(K)_e$ for (H)i exchange is accelerated, more organic anion would appear in the extracellular fluid.

The maximum concentration of cation which can be attained within the cell is limited by the quantity of intracellular non-diffusible anion. Conway[4] has calculated the concentration of the sum of the non-diffusible anions and has represented this sum in terms of the idiomolar value of cell fluid. It is

these large immobile protein phosphate complexes that are important in maintaining the constancy of the total cation concentration of the body fluids. The relative proportion of various cations in the cell is determined by the transfer mechanism described above.

As can be seen from Figure 2, a change in concentration in any one of the components of this exchange system may modify the equilibrium with alteration in muscle or extracellular composition as a result. Since the extracellular concentrations of both potassium and hydrogen are fractions of their intracellular concentrations, a small absolute change in extracellular concentration of these ions must have a considerably greater effect on the equilibrium than the same absolute change in intracellular concentration. Thus, it is the components of low concentration which are of primary importance in the equilibrium. It must be emphasized that these processes are dynamic equilibria in which changes in composition occur only until a new stable state is achieved with altered concentrations of the reactants on each side of the cell membrane. When this new equilibrium is established, the exchange continues at the same rate as before but body composition remains in the same altered state. This altered composition persists until the addition or removal of a reactant again shifts the equilibrium.

Metabolic Alkalosis

In extracellular metabolic alkalosis the fall in extracellular hydrogen ion concentration increases the gradient of hydrogen ion between cell and extracellular fluid. As a consequence, the exchange of $(H)_i$ for $(K)_e$ is accelerated and more $(H)_i$ is transported into the extracellular fluid. The shift in this equilibrium permits the cell to reduce the magnitude of extracellular alkalosis and accounts for the fact that the serum bicarbonate does not rise as high after the administration of a given amount of bicarbonate as calculated from the extracellular distribution of the sodium of administered sodium bicarbonate.[12] The final intracellular hydrogen ion concentration may not be altered or may actually rise since with the reduced breathing which accompanies extracellular alkalosis, the carbon dioxide concentration rises in all body fluids. If intracellular bicarbonate concentration remains constant, as stated by Wallace and Hastings,[20] intracellular hydrogen ion concentration will rise under these circumstances.

When $(H)_i$ for $(K)_e$ exchange is increased the $(Na)_i$ for $(K)_e$ exchange is reduced, because of the competition between these reactions. If it is assumed that the exchange of intracellular sodium $(Na)_i$ for potassium $(K)_e$ is usually of greater magnitude than the exchange of intracellular hydrogen $(H)_i$ for extracellular potassium $(K)_e$, the net result is that less potassium is transported into the cell, and low intracellular potassium results. High intracellular sodium is, of course, the result of diminished sodium transfer outward. The electrostatic equivalent of the non-diffusible

anions within the cell is thus satisfied by low potassium and high sodium. The diminished transfer of potassium into the cell leads to accumulation of potassium in the extracellular fluid since diffusion out of the cell continues at a fairly constant rate. This accumulation of extracellular potassium might account for the increased potassium excretion in the urine observed in metabolic alkalosis,[18] since the kidney assiduously guards against a rise in extracellular potassium concentration.[15]

Metabolic Acidosis

In extracellular metabolic acidosis the rise in hydrogen ion concentration outside the cell decreases the gradient between cell and extracellular fluid. As a consequence, the exchange of $(H)_i$ for $(K)_e$ is decelerated. Such a shift in this equilibrium permits the cell to reduce the magnitude of extracellular acidosis. The exchange of $(Na)_i$ for $(K)_e$ is accelerated, since the competing reaction, $(H)_i$ for $(K)_e$, is reduced. Since $(Na)_i$ for $(K)_e$ exchange is of greater magnitude than $(H)_i$ for $(K)_e$ exchange, intracellular potassium is increased somewhat and intracellular sodium is depressed.

Respiratory Acidosis

In respiratory acidosis the rise in extracellular hydrogen ion concentration decreases the exchange of $(H)_i$ for $(K)_e$. As a consequence, the exchange of $(Na)_i$ for $(K)_e$ is accelerated and intracellular sodium is slightly low or normal while potassium is slightly high or normal. In respiratory alkalosis the converse occurs.

The small magnitude of the changes in muscle composition in respiratory disturbances is readily explained by the fact that carbon dioxide penetrates cells fairly readily and therefore $(H)_i$ varies directly with $(H)_e$ in these states. The $(H)_i$ for $(K)_e$ exchange is not markedly disturbed and therefore the $(Na)_i$ for $(K)_e$ exchange remains relatively unaffected.

The increased undetermined anion concentrations (lactate) observed in states of low extracellular hydrogen ion concentration (alkalosis) may be explained on the basis of greater potassium entry into the cell in exchange for $(H)_i$ rather than $(Na)_i$ and therefore more potassium leaving the cell, presumably as potassium lactate. The increase in serum lactate and fall in muscle potassium observed in exercise is also consistent with this concept since greater $(H)_i$ production would occur under these circumstances.

Potassium Deficiency

In those states in which potassium is lost from the body and the concentration of extracellular potassium falls, there is diminished exchange of both $(Na)_i$ and $(H)_i$ for $(K)_e$. As a result, intracellular potassium falls and intracellular sodium rises. Because of the diminished transfer of hydro-

gen out of the cell, extracellular bicarbonate rises (Fig. 3).[6] In effect the cell adds to the extracellular fluid one fixed cation in excess of fixed anion for each hydrogen ion which accumulates within the cell as the net result of altered transfer. The kidney evidently does not excrete this load of cation without some increased loss of chloride in the urine. This loss of chloride leads to the hypochloremia that is associated with alkalosis in potassium deficiency. It has not been adequately established that the kidney is able to conserve chloride, after bicarbonate loading, less efficiently in potassium deficiency than under normal circumstances.

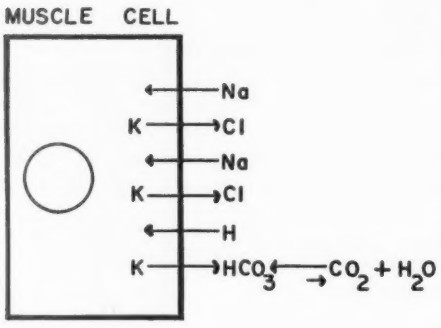

FIG. 3. Mechanism of production of alkalosis in potassium deficiency.

Why the kidney does not compensate for the load of fixed cation in excess of fixed anion which comes from the cells is not known. If the changes in composition of muscle, which are described in potassium deficiency, apply also to the cells of the distal tubule of the kidney, high intracellular hydrogen and low potassium concentration would be present. These findings might account for increased urinary secretion of hydrogen ion in potassium deficiency as postulated by Berliner.[3]

It is of interest to note that if the changes in muscle composition described in this paper apply also to the kidney, the tubular cells of the kidney should show high intracellular hydrogen ion concentration in metabolic and respiratory acidosis and low intracellular hydrogen ion concentration in metabolic and respiratory alkalosis. Urines of low pH would be expected in acidosis and urines of high pH in alkalosis. It is only in the alkalosis associated with potassium deficiency that high intracellular hydrogen ion concentration might be present. Therefore, renal correction of alkalosis in potassium deficiency might be expected to be limited. However, no significant alterations in water and electrolyte content of kidneys of rats, which would confirm these postulations, were noted in potassium deficiency.[9] Small changes in specific cells could not be determined by the method of organ analysis that was used.

The fact that the change in intracellular sodium in potassium deficiency is about twice the change in intracellular hydrogen as derived from determination of muscle sodium and potassium ($\triangle (Na)_i = -\frac{2}{3} \triangle (K)_i$, the

other one-third being Δ $(H)_i$) indicates that the $(Na)_i$ for $(K)_e$ exchange is usually of greater magnitude than the $(H)_i$ for $(K)_e$ exchange. Since $(K)_e$ exchanges for both $(Na)_i$ and $(H)_i$ the reduction of $(K)_e$ would be expected to reduce the transfer of $(Na)_i$ and $(H)_i$ proportionately. For example, if it is assumed that $(Na)_i$ for $(K)_e$ exchange takes place at the rate of 100mM per minute and $(H)_i$ for $(K)_e$ exchange occurs at 50mM per minute and that both reactions are reduced 10 per cent by a fall in $(K)_e$, then, in absolute quantities, the retention of $(Na)_i$ would be twice the retention of $(H)_i$.

In extracellular alkalosis accompanying potassium deficiency, severe intracellular acidosis should be present, since the original disturbance was due to deceleration of hydrogen ion transfer out of the cell. In addition, respiratory compensation of the extracellular alkalosis leads to elevated pCO_2 of the body fluid which further increases intracellular acidosis.

Potassium Excess

The converse of the above processes probably holds true in potassium excess and accounts for the fall in extracellular bicarbonate concentration, the rise in intracellular potassium, and the fall in intracellular sodium which are noted in this condition.[14]

Summary

In summary, the transfer of electrolyte between cell and extracellular fluid and alterations in this transfer may be accounted for in simple physical chemical terms involving the application of the Law of Mass Action. Instead of complex mechanisms requiring specific regulation, the constancy of the cation concentration of muscle would seem to depend in large part upon the constancy of three factors:

1. The intracellular non-diffusible anion concentration is maintained at a stable level through cell anabolism. The absence of variability in muscle phosphorus in many different states (Table 1) is an example of the constancy of these anions. This quantity of anion limits the maximum concentration of cation which can be maintained within the cell. Changes in valence of the phosphate protein complexes can occur only with change in intracellular hydrogen ion concentration. Therefore, with alterations in hydrogen ion exchange, valences of these compounds may be altered (and total intracellular fixed cation concentration may be affected). Because of the free diffusion of water, extracellular concentration of cation parallels that of intracellular cation.

2. The extracellular potassium concentration is regulated within narrow limits by renal transport mechanisms. Thus, alterations in renal function may markedly influence intracellular composition, since the exchange of $(Na)_i$ and $(H)_i$ for $(K)_e$ is altered by change in extracellular potassium concentration.

3. The extracellular hydrogen ion concentration is maintained with little variation by respiratory and renal regulatory mechanisms operating through the buffer systems of the body. The proper and great significance of the extracellular buffers can now be understood since they resist change in extracellular hydrogen ion concentration. This constancy of the extracellular hydrogen ion concentration exerts profound influence on the total body composition by preventing alteration in the exchange of electrolyte between cell and extracellular fluid.

By means of the hypothesis presented in this paper it is possible to predict body electrolyte composition in a variety of disturbances and the data so far obtained from tissue analysis agree with these predictions. This hypothesis in no way conflicts with existing knowledge of the rôle of kidney, lung, or buffers in the regulation of the internal environment of the body but includes them in a dynamic framework operating according to simple thermodynamic principles.

The significance of these concepts is obvious. Body function is dependent upon the transfer of energy within cells and the chemical reactions which release energy depend solely upon the concentrations of reactants, coenzymes, inhibitors, etc. within the cell. The composition of the fluid outside of the cell is of importance only in its effect on composition within the cell. Therefore, since parenteral fluids must be introduced into the extracellular space alone, the fluids administered should alter extracellular concentrations in a way that will correct deviations in intracellular composition.

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RAPID MICRO METHOD FOR THE TURBIDIMETRIC DETERMINATION OF SERUM PROTEIN*

I. A. PARFENTJEV AND M.-L. JOHNSON

The significance of altered serum proteins in disease has long been recognized, but most of the current modes of analysis are either too complicated for routine use or yield unreliable data which are difficult to interpret. In a study of serum proteins for diagnostic purposes, it is essential to have a simple, inexpensive, but accurate procedure for complete protein analysis that will require only a small amount of serum and little time. Since no available technique fulfilled these criteria, we devised a rapid micro method for the turbidimetric analysis of serum which is convenient for clinical application.

The method is based on a photoelectric measurement of the dispersed precipitate produced by $(NH_4)_2SO_4$ in highly dilute solutions of serum. Not only albumin and globulin, but the sub-fractions of globulin are precipitable with varying concentrations of the salt; hence, globulin may be fractionated into gamma, pseudo, and euglobulin with equal facility. When certain additional studies were made concerning the separation of acid insoluble proteins derived mainly from the globulin fractions, it was found that these were precipitable in acetate buffer at pH 5.1 and 6.0. In every instance, despite the method of precipitation, the protein concentration was estimated from the observed turbidity.

MATERIALS AND METHODS

The solutions of $(NH_4)_2SO_4$ used for precipitation of the various protein fractions were prepared according to the findings of Cohn and others.[1,2,4,5] Table 1, which shows the components of one liter of each salt solution, also lists the protein fraction that can be expected to precipitate at that $(NH_4)_2SO_4$ concentration, when a sample of serum is successively treated with increasingly stronger solutions. It should be noted that each preparation, brought to a volume of one liter with distilled H_2O, contained 1% NaCl and enough gum ghatti to serve as a protecting colloid.

Sufficient 10 N NaOH was added to each preparation of $(NH_4)_2SO_4$ to bring the pH to about 7 as indicated by 0.02% phenol red. To prevent microbial growth, the preservative merthiolate was added to all solutions in a dilution of 25 ppm., save the 50% preparation, where it was deemed unnecessary because of the high salt concentration. The salt preparations were permitted to stand overnight, then filtered free of undissolved gum ghatti.

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With these $(NH_4)_2SO_4$ solutions, we produced a suspension of precipitated protein and measured the turbidity, at first on a Klett-Summerson photoelectric colorimeter and subsequently on a Coleman Universal Spectrophotometer. Calculations are based on the transmittance at a known wave length of light, 450 λ, where the blank salt solution is adjusted to 0 density, and most of the turbid samples show a 20-60% increment in optical density reading.

All sera analyzed were limpid, with persistent debris removed by repeated contrifugation. When either chyle or hemolysis was evident, a blank of the serum diluted in NaCl was read against NaCl and a correction made by subtracting the optical density of serum in saline from the final reading made with $(NH_4)_2SO_4$.

TABLE 1

VARIOUS $(NH_4)_2SO_4$ SOLUTIONS USED FOR PRECIPITATION OF SERUM PROTEIN FRACTIONS

% Solution of $(NH_4)_2SO_4$	$(NH_4)_2SO_4$ *in grams*	NaCl *in grams*	*Gum ghatti* in grams*	*Distilled* H_2O	*Protein fraction precipitated*
18	183.67	10.0	5.5	to 1 liter	largely gamma globulin
21	216.07	10.0	5.5	to 1 liter	gamma, beta, alpha globulin
27	270.80	10.0	5.5	to 1 liter	beta, alpha, mucoglobulin
50	500.00	10.0	7.0	to 1 liter	albumin

* Coleman & Bell Company, Norwood, Ohio.

Our procedure has been to pipette the required quantity of serum or protein solution into optically ground round cuvettes (19 x 105 mm.). For pipetting we have used the blue-line Kahn 0.2 ml. pipettes and, recently, the more precise transfer Kirk type† of a 0.02 ml. capacity. With such minute amounts of material it was necessary to add 0.1 ml. of physiological saline to protect the protein from drying or forming large flocculating particles upon contact with the precipitating salt. Ten ml. of the $(NH_4)_2SO_4$ solution were buretted into each tube; the mixture was well shaken and a reading was made on the spectrophotometer. A recent modification has indicated that greater precision may be achieved by using dilution techniques in which larger volumes are involved. In either case the specific $(NH_4)_2SO_4$ solution serves as the blank and increasing concentrations of protein register as increasing optical densities.

To provide a convenient outline of our procedure we have tabulated in Table 2 the quantities of serum which are analyzed with each concentration of $(NH_4)_2SO_4$. Included with the actual amounts of serum analyzed are the appropriate quantities of diluted material as well as the dilutions which we have found practical and more precise for wide scale testing.

† Microchemical Specialties Company, Berkeley, California.

Interpretation of turbidimetric readings

From pilot tests with sera of known protein concentration, it was found that precipitation with 50% $(NH_4)_2SO_4$, which is used for the determination of total protein, required 0.02 ml. of the serum sample to give a convenient optical density reading on our spectrophotometer. Similarly, 0.05 ml. of serum was needed for a corresponding optical density with the 27% concentration of the salt which precipitates all globulin.[1] Using these quantities of serum for total protein and total globulin, the turbidity readings varied directly with the protein content of each serum and it was established that the observed turbidity is a function of concentration.

TABLE 2

OUTLINE OF PROCEDURE: AMOUNT OF SERUM ANALYZED AT EACH SALT CONCENTRATION

Protein analyzed	$(NH_4)_2SO_4$ *concentration* (10 ml.)	*Amount of serum analyzed*	*Preferred dilution*	*Amount of dilution analyzed*
Total protein	50%	0.02 ml.	1-6	0.12 ml.
Total globulin	27%	0.05 ml.	1-3	0.15 ml.
Euglobulin	21%	0.05 ml.	1-3	0.15 ml.
Gamma globulin	18%	0.10 ml.	no dilution	

However, if the specified amounts of a protein solution gave optical densities below or beyond the range of our spectrophotometer (optical density 0.15 to 0.40), we substituted an amount that would be readable, and by the appropriate correction for volume calculated the actual concentration of the solution.

When investigating the progressive increase of optical density that parallels increasing protein concentration, sera of known protein content were taken in amounts calculated to give turbidity readings corresponding to 4, 5, 6, 7, and 8 grams per 100 ml. when precipitated with the 50% salt solution, and in amounts that would correspond to 2, 3, and 4 grams when precipitated with the 27% concentration. The optical densities of all these dilutions were read, averaged, and plotted against the protein concentration and were shown to have a straight line relationship (Figs. 1 and 2).

When these data were used to derive the slope of the line ($y = a + bx$), "a," in each instance, was insignificant and considered equal to zero for all practical purposes. Hence, if the known protein concentration of a serum, expressed in gm./100 ml., is divided by the observed optical density reading, a constant factor is the quotient. The factor for each sample in Figures 1 and 2 was calculated and the average factor for each salt concentration was

established (ref. Table 3). Thereafter, the protein fraction of unknown sera could be determined by simple multiplication of the observed optical density (OD) by the specific factor for total protein (K_{TP}) or total globulin (K_{TG}) as the case might be.

This procedure for establishing a factor has been repeated with many sera of known protein concentration, comprising 495 analyses. The per cent error of each sample was determined by dividing the concentration difference of the two methods, turbidity and a standard procedure, by the known concentration, and the coefficient of variation was found to be less than 6%.

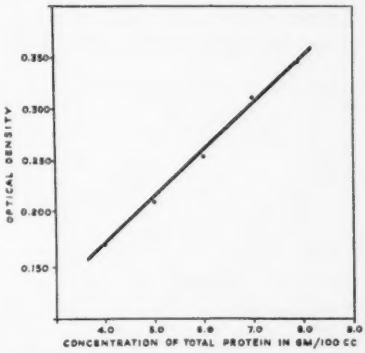

FIG. 1. Relationship of optical density to increasing concentrations of total protein.

Standardization of analyses

The above data indicate that establishing a factor by a technique familiar in colorimetry is quite applicable to turbidimetric studies of dilute protein solutions in $(NH_4)_2SO_4$. Our calculations are quite simplified, however, because the amount of serum analyzed is constant for any one salt concentration and it is posible to dispense with a consideration of volume. Furthermore, one factor suffices for each $(NH_4)_2SO_4$ solution since the concentration of any specific fraction varies within comparatively narrow limits. Nevertheless, these factors will vary with each spectrophotometer and electrical source used and from one salt concentration to another. Adjusting a photoelectric cell against a Didymium filter of known optical density is not sufficient; in addition, the instrument must be calibrated and checked against protein solutions of known concentration. For this purpose and for calculating factors with other salt concentrations we have used both whole human sera previously analyzed by accepted procedures as well as special standards prepared from purified protein.

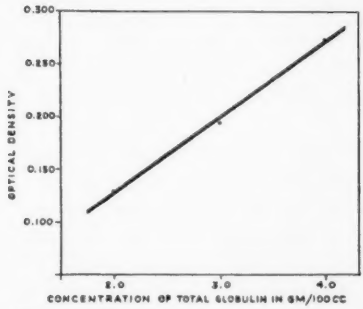

FIG. 2. Relationship of optical density to increasing concentrations of total globulin.

*Reference sera of known analysis**

Basic among our standard protein solutions are the sera analyzed for nitrogen by the Kjeldahl method, where multiplying by the appropriate factor (6.25) gives a direct estimate of the total protein present. To analyze for protein fractions, Majoor[5, 6] suggested Na_2SO_4 in a concentration of 268 gm./liter which will separate albumin from globulin. After precipitating the total globulin with salt, the supernatant is analyzed for protein, again by the Kjeldahl method, and the albumin concentration thus determined. By subtraction from total protein, the pattern of albumin and globulin in a given serum sample can be established.

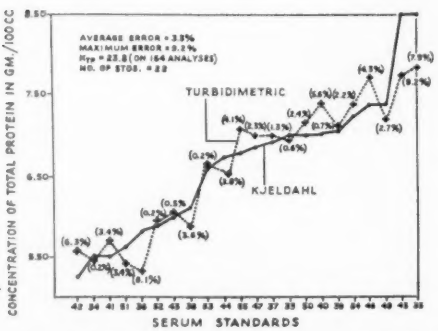

FIG. 3. Comparison of turbidimetric determinations (161) with Kjeldahl values for total protein. In each instance, the turbidimetric concentration is indicated by +, the Kjeldahl value by •; the per cent error, expressed in parenthesis, was determined by dividing the concentration difference of the two methods by the Kjeldahl concentration. The numbers at the bottom of the figure merely refer to specific serum standards, 22 in all.

In comparing our turbidimetric determinations of protein concentration with the known Kjeldahl values, we have plotted our results with 42 standards (Figs. 3 and 4). For convenience, the serum standards were arranged in order of increasing concentration, spanning a total protein range of 5-8 gm./100 ml. and a globulin range from 2-6 gm./100 ml. From these data it is evident that the precision of the method was not favored at any one level rather than another.

With 22 standards of known total protein content, the per cent error of turbidimetric analyses ranges from 0.2% to 9.2% with an average error of 3.3%. The coefficient of variation is 4.21%, which means that two-thirds of all analyses for total protein will deviate from the Kjeldahl value by less than 4%, 95% of analyses by less than 8% (Fig. 3). Similarly, 20 standards of known total globulin, determined by subtracting Kjeldahl albumin from Kjeldahl protein, gave an average error of 5.7% with a 0.4% to 11.2% range and a coefficient of variation of 6.35%. With globulin, therefore, two-thirds of all analyses will deviate from the Kjeldahl value by less than 6%; 95% will have an error less than 12% (Fig. 4). In subsequent tests, more than 150 reference sera were checked against

* Generously provided by Dr. J. P. Peters' laboratory with the kind co-operation of Miss Hald.

Kjeldahl values and the difference between turbidity and Kjeldahl was always within the range described. It should be noted that the error of the Kjeldahl method itself was never taken into account, but was accepted as a standard procedure of reference.

Protein standards

Specific protein fractions, dissolved in physiological saline to calculated concentrations, provide stable standards of reference for establishing factors as well as for estimating experimental error. These preparations of purified protein are adequate substitutes for sera of known constitution and have proved to be equally precise, quite stable, and much more convenient.

Establishing factors. To check the appropriate factor for a specific concentration of $(NH_4)_2SO_4$, for example, pure proteins were taken in known amounts and precipitated with the 50% or 27% solution of salt so that the individual factors might be determined and averaged. In one experiment with human serum albumin provided by the American National Red Cross, the stock solution had a protein concentration of 25 gm./100 ml. as checked by Kjeldahl analysis. From this, multiple dilutions were made to cover a protein range of 1.25 to 8.75 gm./100 ml.

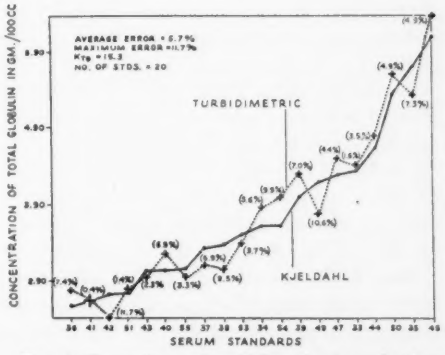

FIG. 4. Comparison of turbidimetric determinations (113) with Kjeldahl values for total globulin. In this figure the turbidimetric concentration is again indicated by +. The Kjeldahl •, however, means that the albumin was analyzed by the Kjeldahl method, as was the total protein, while the globulin was determined by subtraction. The per cent error, expressed in parenthesis, was determined by dividing the concentration difference of the two methods by the Kjeldahl concentration. The numbers at the bottom of the figure refer to specific serum standards, a total of 20.

A study with human albumin could be made only with the 50% solution of $(NH_4)_2SO_4$, while precipitation of other pure fractions prepared from serum globulin permitted extensive investigation and comparison involving the various concentrations of $(NH_4)_2SO_4$. In subsequent studies, therefore, we worked with several pure protein fractions such as Fraction II of Cohn.* A solution of this gamma globulin in NaCl was checked for protein content by the Kjeldahl technique so that the known material could be taken in sufficient quantities to give a range of protein concentration similar to the

* Kindly supplied by Dr. Cohn's laboratory, courtesy of Dr. Surgenor.

albumin standards. Other protein fractions precipitated with varying concentrations of $(NH_4)_2SO_4$ and included in this series were immune globulin from the Pitman-Moore Company, which can be shown to be 16% globulin, tetanus antitoxin (1500 units/ml.) from the Eli Lilly Company, which is 10.54% protein and diphtheria antitoxin* (20,000 units/ml.) from Lederle, which is 18% protein.

From the data gathered with these protein standards it was established that the factor for 50% $(NH_4)_2SO_4$ is 24.3 based on 194 analyses, and 13.9 for the 27% concentration, based on 102 analyses. These figures closely approximate the factors obtained with serum samples of known protein content, and the over-all average from data on 901 analyses indicates a total protein factor (K_{TP}) of 23.8 and a total globulin factor (K_{TG}) of 15.5.

From the data in Table 3 it can be determined that the average factor is 23.8 for 50% $(NH_4)_2SO_4$. This is a weighted average accounting for each factor with each protein fraction, a total of 495 analyses in all. Similarly, the weighted average factor for the 27% salt concentration is 15.5 based on 406 individual calculations. The average deviation of the factors from one protein to another has an equivalent protein value of 0.12 gm./100 ml. for the 50% solution and 0.24 gm./100 ml. for the 27% preparation.

Despite the variety of protein standards used, the uniformity of results attests to the applicability and usefulness of pure protein standards. Apparently, the kind of precipitate and degree of turbidity depend upon the salt concentration rather than the size and molecular weight of the protein particle.

Determination of acid insoluble proteins. Until now, the fractions of serum protein which we have considered have been studied by many and are familiar to most. The techniques of fractionation are arbitrary methods that have been worked out for separating the various components of serum for purposes of study. In another phase of our studies, we have chosen to split serum protein into fractional components by other than the conventional methods, directing our attention to certain serum proteins, insoluble in acidulated H_2O. These acid insoluble proteins are present in all sera being derived mainly from the globulin fractions, and we have found them to be of diagnostic significance which will be reported in detail in a subsequent paper. However, since our method of analyzing these proteins is based on turbidity, we feel that a description of the procedure should be included in this report.

It was found that when 0.05 ml. of serum was added to 10 ml. of acetate buffer at pH 5.1 or at 6.0, a readable turbidity registered on the Coleman spectrophotometer. To determine the concentration of the acid insoluble proteins, larger quantities of serum were precipitated at each pH, centrifuged in graduated tubes, and the precipitate was redissolved at neutrality

* Received through the kindness of Dr. Henry Piersma of Lederle Laboratories.

TABLE 3

COMPARISON OF FACTORS OBTAINED WITH VARIOUS PROTEIN SOLUTIONS AT SPECIFIC $(NH_4)_2SO_4$ CONCENTRATIONS

	with 50% $(NH_4)_2SO_4$					*with* 27% $(NH_4)_2SO_4$				
Protein solution	*No. of analyses*	*Sum of factors*	*Factor*	*Deviation from 23.8 (Av. factor)*	*Deviation as protein*	*No. of analyses*	*Sum of factors*	*Factor*	*Deviation from 15.5 (Av. factor)*	*Deviation as protein*
Serum total protein	301	7,058.6	23.5	0.3	0.06	...	.....	...	..	...
Human albumin	128	3,161.6	24.7	0.9	0.18	...	.....	...	..	...
Serum total globulin	...	.....	...	..		304	4,865.8	16.0	0.5	0.20
Gamma globulin	48	1,118.4	23.3	0.5	0.10	48	624.0	13.0	2.5	0.50
Immune globulin	18	442.8	24.6	0.8	0.16	18	255.6	14.2	1.3	0.26
Tetanus antitoxin	...	.....	...	..	...	12	172.8	14.4	1.1	0.22
Diphtheria antitoxin	...	.....	...	..	...	24	369.6	15.4	0.1	0.02
Total	495					406				

in 7/10 of the original serum volume. The actual protein content of the concentrated material was determined by turbidity with 50% $(NH_4)_2SO_4$ and the average concentration of acid insoluble proteins in serum was calculated to be about 0.5 gm./100 ml. ranging from 0.3 to 0.7 grams.

With sera having acid fractions of known protein content as determined above, it was possible to calculate each acid fraction constant by dividing the protein value by the optical density observed with 0.05 ml. of serum at each pH value. In all, 12 standards were analyzed in duplicate at both pH 5.1 and pH 6.0 and an average factor of 1.93 was obtained.

Estimating instrumental and subjective error. Apart from the primary advantage of establishing a factor, the stability of our suspensions of protein precipitate in $(NH_4)_2SO_4$ makes it possible to estimate the instrumental error due to hourly and daily fluctuations of the spectrophotometer. In this study, repeated readings were made of two series of protein standards.

In the first set the same human serum albumin in a concentration of 25 gm./100 ml. was diluted 1-10 and taken in duplicate amounts of 0.03 and 0.05 ml. This was equal to a protein concentration of 3.75 gm. and 6.25 gm., respectively, when 50% $(NH_4)_2SO_4$ was added and the resulting optical density multiplied by the total protein factor. The precipitated material, stoppered in cuvettes and thoroughly shaken before each reading, was reread over a period of one month. A total of 71 readings was made with an average deviation of $\pm$ 0.25 gm. of protein.

The second series of standards was with gamma globulin where the known material was taken in duplicate amounts of sufficient quantity (0.12, 0.15, and 0.19 ml.) to give a protein level of 2.5, 3.2, and 4.0 gm./100 ml. when precipitated with 27% $(NH_4)_2SO_4$. In each instance the amount of protein was the product of the optical density and the factor corresponding to the salt concentration. The six samples were precipitated with either 27% or 50% $(NH_4)_2SO_4$ as required, stoppered in cuvettes, and reread 96 times over a one month period. The average deviation was $\pm$ 0.21 gm. of protein for 27% $(NH_4)_2SO_4$, and $\pm$ 0.53 gm. for 50% $(NH_4)_2SO_4$. From this it can be calculated that the average instrument error for total protein is 6.5%, for total globulin 6.6%.

The described rereadings of precipitated standards day after day indicates the stability of our spectrophotometer. Values, calculated from these repeated samplings of stock solutions of protein as well as sera of known analysis, show reproducible results with an average error of less than 7%. To study the subjective error of the individual worker, we compared data from the same serum samples obtained by different people at different times. On the basis of 48 analyses, the average deviation for total protein was 2.7%, for total globulin 4.4%.

Before concluding this section, several advantages of protein standards should be emphasized. First and foremost, the use of pure protein fraction

frees the turbidimetric method from dependence on any other analytical procedure. In addition the spectrophotometer of choice may be readily standardized with solutions of purified protein and because of the stability of the dispersed precipitate, these standards may be sealed in cuvettes and kept indefinitely. Solutions of known protein content are also useful for evaluating comparative analyses to determine subjective error.

Consideration of nephelometry

Investigating the possible use of nephelometry as a more precise procedure for the measurement of precipitated protein, we have used the Coleman Photo-nephelometer and have analyzed several sera that had been fractionated by the Na_2SO_4-Kjeldahl technique. In order to use protein material in quantities that are identical with those analyzed by turbidimetry, it was necessary to dilute the serum for nephelometry, 1:40 for total protein, 1:8 for total globulin. Thus, for an average total protein of 6.5 gm./100 ml., it may be calculated that the actual protein analyzed was 0.00016 gm.; for an average total globulin of 2.7, 0.00038 gm.

Estimation of serum total protein by this modification gave an average error of 5.7%, based on 18 analyses. When total globulin, diluted 1:8, was analyzed in a similar way, the average error was 10%. Although nephelometry is sensitive to more minute amounts of protein than is turbidimetry, our pilot studies are too few to permit a proper evoluation of the instrument for clinical use in the fractionation of serum.

The rapid micro method compared to accepted procedures

Comparison with the Kjeldahl analysis for protein. Sera analyzed by the Kjeldahl method have been used as one series of reference standards for our rapid micro method, and therefore the comparison between turbidity and Kjeldahl is considered in detail under "Standardization of Analyses." It will suffice, then, to recall that the average difference between the two methods is less than 5%, and it may be concluded that the precision of our method falls within the limits prescribed for routine analyses.

Since an estimation of albumin by either the Kjeldahl or turbidimetric method is dependent on the precipitation of globulin, it is interesting to compare the various salt concentrations used. The $(NH_4)_2SO_4$ solution recommended by Cohn[1] for the precipitation of total globulin is 270 gm./liter, which closely approximates the 268 gm./liter of Na_2SO_4 used by Majoor[7] and the 280 gm./liter of Na_2SO_3 used by Wolfson.[10] In our experience, separation of total globulin by 270 gm./liter of $(NH_4)_2SO_4$ is quite clear cut, and the concentration of the salt must be increased by at least 10% before any albumin is precipitated.

We have not compared turbidity with the Biuret Method since analyses by Biuret are often found to deviate from reference Kjeldahl values by as

much as 10%. Nevertheless, it is worth noting that Kibrick and Blonstein,[8] using Na_2SO_4 and Biuret, subfractionated serum globulin into alpha, beta, and gamma globulin.

Comparison with electrophoretic data. The average of our analyses of normal human sera gives a protein pattern which is in close accord with the normal electrophoretic patterns described by Petermann and Hogness[9] and by Mider, *et al.*[8] Table 4 indicates the average values determined by these two series of electrophoretic studies and compares them with the average protein pattern established by our turbidimetric technique. However, to compare these electrophoretic analyses of plasma with our data on serum, it was necessary to subtract each author's value for fibrinogen from his total protein and total globulin and to make a corresponding adjustment of the albumin-globulin ratios.

TABLE 4

ELECTROPHORETIC AND TURBIDIMETRIC ANALYSES OF NORMAL HUMAN SERA

	Total prot.	*Total glob.*	*Gamma glob.*	*Albumin*	*A/G ratio*
Electrophoretic—Mider, *et al.*[8]	6.52*	2.48*	0.66	4.04	1.63*
Electrophoretic—Petermann, *et al.*[9]	6.65*	2.62*	0.74	4.03	1.54*
Turbidimetric (74 sera)	6.77	2.60	0.64	4.17	1.60

* Calculated from the authors' data by subtraction of their value for fibrinogen.

It should be noted that Mider found an 8% discrepancy between his electrophoretic values and those obtained by the Na_2SO_4-Kjeldahl procedure, a somewhat greater error than that which occurred with our method.

Comparison with related turbidity studies. Separating the protein components of serum with salt has been an accepted procedure for years and analysis based on turbidity is not new, but the application of the combined techniques to serum fractionation has been limited to a few studies with globulin. In 1939, Looney and Walsh[6] described a turbidimetric estimation of globulin using 1 ml. of a 1 : 10 dilution of serum in 3 ml. of fully saturated $(NH_4)_2SO_4$ and 2 ml. of 2% gum ghatti. This would make their $(NH_4)_2SO_4$ concentration approximately 25%, which is somewhat less than that generally accepted for the separation of total globulin.[1] Although these workers also determined total protein by turbidity, it was precipitated with 5% sulfosalicylic acid.

Recently, Huerga and Popper[5] have adapted the procedure used in the thymol turbidity test to measure serum gamma globulin. Using 18.9% $(NH_4)_2SO_4$ in a 2.93% solution of NaCl without colloid, they found an

average difference of 15% between their method and electrophoretic studies. With a similar technique, Levin, *et al.*[4] estimated serum gamma globulin by turbidity using 19.3% $(NH_4)_2SO_4$, a slightly higher concentration than that used by the preceding workers. Their data indicate good correlation between turbidimetric values and micro-Kjeldahl analyses.

Conclusion

A rapid and convenient method for the complete fractionation of serum protein has been devised. Applicable to all serum protein components, the rapid micro method permits analyses which deviate less than 5% from Kjeldahl values, and provides data in close accord with electrophoretic studies. In contrast to Na_2SO_4 and the Kjeldahl procedure, the use of $(NH_4)_2SO_4$ and turbidity requires no lengthy filtration at elevated temperatures and no elaborate and time-consuming analyses for nitrogen. Compared to electrophoresis, the simplicity of technique and nominal cost of our method are distinct advantages. Scores of duplicate analyses can be accomplished within one day, but apart from speed and convenience as well as economy, the amount of serum required is a small fraction of that demanded by the more elaborate procedures. Furthermore, the turbidimetric method can be constantly checked against standards of known protein concentration where the pure fraction has been dissolved in varying amounts. Hence, there need be no dependence on other analytical procedures.

All in all, the consistency, accuracy, and simplicity of this rapid micro method for serum fractionation emphasize the possibility of extensive clinical use as a facile routine procedure. Moreover, the method is applicable to the study of aberrant protein patterns, as will be shown in a subsequent paper.

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CIRCULATORY CHANGES IN PULMONARY ARTERIO-VENOUS FISTULA*

FRANK D. GRAY, JR., PAUL R. LURIE,** AND RUTH WHITTEMORE

Reports of vascular tumors of the lung including arterio-venous fistula have appeared in the medical literature periodically since 1897.[6] Perhaps because of the stimulus of newer diagnostic techniques, there has been a recent increase in the number of case reports and reviews published.[1, 9, 21, 24] The clinical features and diagnosis of pulmonary arterio-venous fistula have been amply discussed in these reviews; therefore, the emphasis in the present report will be on the pathological physiology. The observations to be presented were made on four patients who were studied during the past two years in the Grace-New Haven Community Hospital.

Interest in this laboratory has been centered about changes in the circulation of the lung associated with cardiovascular and pulmonary diseases. These changes have been found to be, for the most part, a shunting of blood through inaccessible (non-aerated) pulmonary capillaries,[11] and the development of an extensive collateral circulation between the systemic arteries and the pulmonary arteries.[11, 18] Since most pulmonary fistulas consist of abnormal blood channels connecting the pulmonary artery to the pulmonary vein, thus by-passing the accessible (aerated) pulmonary capillaries, it is apparent that physiological studies on patients with these lesions would reveal an inaccessible pulmonary flow. Indirectly such an inaccessible flow has been demonstrated in previously reported cases in which incomplete oxygen saturation of the arterial blood had been found.[1, 5, 17] The presence of collateral circulation between the systemic arteries and the pulmonary artery in arterio-venous fistula has not been reported.

Earlier studies of collateral pulmonary blood flow in other diseases have suggested inflammation,[11, 18] differences in systemic and pulmonary artery pressures,[8] and anoxemia[4] as the initiating factors in the development of these abnormal channels. Since lowered arterial blood oxygen saturation is characteristic of many cases of pulmonary fistula, it seemed of interest to find out whether collateral pulmonary circulation had developed in some.

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Methods

The techniques and formulas used in this study have been reported in detail elsewhere.[11] They consisted of arterial oxygen saturation studies made during the breathing of room air followed by 100% oxygen, cardiac catheterization for the determination of the blood oxygen content of mixed venous blood from the right side of the heart, and the application of previously described mathematical relationships to these data for the purpose of deriving values for blood flow.[11, 22]

Total pulmonary capillary flow was estimated in one case by an indirect method involving carbon dioxide equilibration which has previously been reported in detail.[10] Modifications of the indirect method recently introduced in this laboratory[13] have given a range of variation, compared to the direct Fick method, of ±15%. The difference between total pulmonary capillary flow and systemic flow is an approximation of the collateral bronchial to pulmonary artery flow.[8] These studies were done under basal conditions on consecutive days.

Table 1

	Former Notation	Standard Notation
1.	$S.F. = \frac{O}{a - v}$	$\dot{Q}b = \frac{\dot{V}_{O_2}}{Ca_{O_2} - C\bar{v}_{O_2}}$
2.	$\alpha = \frac{(T+d-LT)-a}{(T+d-LT)-v}$	$\alpha = \frac{CL\bar{c}_{O_2} - Ca_{O_2}}{CL\bar{c}_{O_2} - C\bar{v}_{O_2}}$
3.	$\alpha_o = \frac{(T+d-a)_o}{(T+d-a)_o + (a-v)}$ [22]	$\alpha_o = \frac{(Tb_{O_2}+Cbd_{O_2}-Ca_{O_2})_o}{(Tb_{O_2}+Cbd_{O_2}-Ca_{O_2})_o + (Ca_{O_2}-C\bar{v}_{O_2})}$
4.	$L = \frac{(a_o - a_{a_{1r}}) - (T_o - T_{a_{1r}}) - (d_o - d_{a_{1r}})}{T_{a_{1r}}}$ [23]	$L = \frac{(Ca_{O_2o} - Ca_{O_2}) - (Tb_{O_2o} - Tb_{O_2}) - (Cbd_{O_2o} - Cbd_{O_2})}{Tb_{O_2}}$

S.F. = $\dot{Q}b$ = Systemic blood flow in Liters/minute; O = $\dot{V}_{O_2}$ = Oxygen used in cc./minute; a = Ca_{O_2} = Arterial blood oxygen content in volumes per cent × 10; v = $C\bar{v}_{O_2}$ = Mixed venous blood oxygen content in volumes per cent × 10; α = Net right-to-left shunt of blood in per cent of systemic blood flow; T = Tb_{O_2} = Total corrected blood oxygen capacity in volumes per cent × 10; d = Cbd_{O_2} = Oxygen physically dissolved in blood in volumes per cent × 10; L = Per cent of total hemoglobin passing through ventilated pulmonary capillaries without becoming oxygenated; (T + d — LT) = $CL\bar{c}_{O_2}$ = Expression denoting pulmonary capillary blood oxygen content in volumes per cent × 10.[23] Subscripts $_o$ and $_{a1r}$ refer to functions estimated while breathing 100% oxygen and room air respectively.

The decrease in arterial oxygen saturation due to an increased alveolar oxygen diffusion gradient is overcome by breathing 100% oxygen. However, pure oxygen will only partially overcome a deficit due to shunting of mixed venous blood into the pulmonary vein such as occurs in pulmonary arterio-venous fistulas.[8] The primarily ventilatory causes of an arterial oxygen saturation deficit are called the "L" factor of Lundsgaard and Van Slyke,[18] whereas the inaccessible blood flow or right-to-left shunt is contained in their "α" factor. This factor represents the "net" or algebraic sum of right-to-left and left-to-right shunts between the venous and arterial blood circulations. In a series of cases of chronic pulmonary disease, previously reported,[11] the breathing of pure oxygen tended to increase α, and this finding was used as a basis for calculating the amount of left-to-right (bronchial to pulmonary artery) collateral blood flow. However, recent reports of the occurrence of significant enlargement of the bronchial venous system in some cases of pulmonary disease[15, 19] make such calculations of dubious validity; hence, no attempt has been made to use them in the present report.

In order to conform to the standard terminology recommended by a group of American physiologists,[7] a comparison of our previously used symbols and their equivalents in the new terminology is given in Table 1.

TABLE 2
CLINICAL DATA

Patient	*Age*	*Sex*	*Cyanosis*	*Clubbing*	*Hemoglobin*	*X-ray*	*Bruit*	*Related data*
D.K.	55	F.	0	++++	11.0 G. %	RLL LLL	+	1. Chronic osteomyelitis. 2. Epistaxis. 3. Telangiectasias of lips. 4.? Familial history.
H.C.	54	F.	0	++	16.5 G. %	RLL	+	1. Hypertension. 2. Epistaxis. 3. No visible telangiectasias. 4. No familial history. 5. RLL lobectomy performed.
A.B.	32	M.	0	0	15.0 G. %	LUL	+	1. Rare epistaxis. 2. No visible telangiectasias. 3. No familial history. 4. LUL lobectomy performed.
S.K.	57	F.	++++	++++	18.8 G. %	RLL	Absent	1. Rheumatic mitral stenosis; auricular fibrillation; congestive failure. 2. No visible telangiectasias. 3. No familial history. 4. RML; RLL lobectomy performed.

RESULTS AND DISCUSSION

The clinical findings in the four cases of pulmonary arterio-venous fistula are summarized in Table 2. In only one patient, D.K.,* was evidence of associated telangiectasias clearly observed. The absence of polycythemia in this patient may have been related to a long-standing extensive osteomyelitis.

* Dr. S. D. Kushlan has prepared a detailed clinical report of this patient.[10]

Cyanosis was not conspicuous in the present group, and, as may be seen in Table 4, a resting arterial blood oxygen saturation in the range in which cyanosis might be expected was found in only one patient, S.K.

The only consistent clinical finding observed in all four patients was an x-ray picture which could be called typical of pulmonary arterio-venous fistula.

TABLE 3
OBSERVED DATA

Patient	*Oxygen used cc./min.*	*Blood oxygen content, vol. %*			*Arterial dissolved oxygen (air) vol. %**	*Corrected oxygen capacity vol. %*
		Resting arterial	*Resting venous*	*Arterial breathing oxygen*		
D.K.	215	11.9	8.6	13.6	0.17	13.4
H.C.	237	18.9	12.9	20.8	0.16	20.6
A.B.	245	18.8	15.2	20.3	0.24	19.6
S.K.	192	17.9	...	19.3	0.13	22.6

* Dissolved O_2 while breathing 100% O_2 assumed to be 1.0 vol. %.[23]

TABLE 4
DERIVED DATA

Patient	*Arterial blood oxygen saturation, %*		Decimal fraction × 100		*Blood flow, L/min.*	
	Resting	*Breathing 100% oxygen*	*"net" % a or a_o*	*"L" %*	*Systemic*	*Pulmonary capillary*
D.K.	88	94	19.5	6.5	6.5	...
H.C.	91	96	11.8	5.2	3.95	...
A.B.	95	96	7.7	3.8	6.8	10.2
S.K.	79	81	42 to 59*	2.3	...	...

* Since a resting venous blood oxygen value was not determined in this case, a_o was calculated by formula 3, Table 1, using an arterio-venous difference range of 3 to 6 vol. % within which most cases fall.

The physiological findings are summarized in Tables 3 and 4. The expected right-to-left, a, shunt of pulmonary arterial blood into the pulmonary vein was found in all cases. Furthermore, in the one case, A.B., in which the collateral flow between systemic and pulmonary arteries could be approximated, the presence of a large flow through these channels was evident. Such abnormal channels were not found in the resected specimen, but the local nature of the resection might have allowed large numbers of diffuse, small collateral channels to escape attention. The findings in this case appear, at first glance, to be contradictory. Clinically there was no cyanosis, and a 95% oxygen saturation was found on analysis of the

peripheral arterial blood. These factors do not seem consistent with a right-to-left shunting of mixed venous blood through a pulmonary arterio-venous fistula. In spite of this, a typical pulmonary artery to pulmonary vein fistula was found at operation. The only physiological finding consistent with the pathological specimen was the failure of the arterial blood oxygen saturation to rise to 100% on breathing pure oxygen. When the formula for calculating the a shunt on the basis of this test is applied, a relatively small net right-to-left intra-pulmonary shunt of 7.7% of the total systemic flow is derived. This amounts to a net right-to-left shunt of 0.5 L/min. which, in the presence of normal arterial oxygen saturation calls for an analysis in terms of factors known to influence the oxygen saturation of arterial blood. Most of these factors have been clearly pre-

TABLE 5

SHOWING EFFECT OF COLLATERAL CIRCULATION ON ARTERIAL OXYGEN SATURATION AND LEFT VENTRICULAR WORK AS ESTIMATED BY LEFT VENTRICULAR OUTPUT

Collateral flow L/min.	*Oxygen used cc./min.*	*Blood oxygen capacity vol. %*	*Resting venous blood O_2 vol. %*	*Decimal fraction × 100 (%)*		*Resting arterial blood O_2 vol. %*	*Arterial oxygen saturation %*	*Systemic blood flow L/min.*	*Left ventricular output L/min.*
				"L"	*"a"*				
None	200	20.0	15.0	5.0	10	18.82	93	5.25	5.25
2.0	200	20.0	15.0	5.0	10	18.99	94	5.0	7.0

sented by Lundsgaard and Van Slyke.[10] The chief ones which decrease oxygen saturation are, on the one hand, a shunting of venous blood into the arterial tree (a factor), and on the other, a decrease in the ability of the lung to bring the oxygen of inspired air into contact with the pulmonary capillary blood (L factor). The latter proved to be minimal in case A.B. (Table 4). The former, in terms of "net" shunt was also small. However, a small "net" right-to-left shunt in the presence of a large collateral left-to-right shunt is indicative of a much larger "total" right-to-left shunt.

This collateral left-to-right shunt between systemic and pulmonary arteries is a further factor which influences the arterial oxygen saturation. Its effect has been demonstrated in a previous report.[11] A large blood flow through these channels tends to raise the arterial oxygen saturation. Although this factor might conceivably raise the resting arterial oxygen saturation value to the normal range, there would always be a deficit from the expected value of 100% following the breathing of pure oxygen, unless the collateral flow were many times greater than the right-to-left shunt. The precise effect of collateral flow on the arterial saturation in this case cannot be determined because the volume can be calculated only approximately. However, by using average normal values for oxygen consumption and blood gas contents, the effect of a large collateral shunt can be shown for a hypothetical case having a moderate a shunt (Table 5). The effect would be

greater in cases with larger a shunts. The enormous increase in output required of the left ventricle to maintain the collateral flow necessary to produce a small increase above the expected value of arterial oxygen saturation by this means is indicated in Table 5. From the standpoint of cardiac work involved this does not appear to be an efficient means of overcoming arterial anoxemia of the degree found in this group of cases.

The age of patients D.K. and H.C. places them in a group in which chronic lung disease with its concomitant development of collateral circulation often occurs. However, there is no reason to believe that case A.B. had associated lung disease. It seems possible, therefore, that early in his development, a lowered blood oxygen tension, produced by the shunting of venous blood into the pulmonary vein, was responsible for the appearance of collateral flow in this case.

If arterial anoxemia, present from birth in some cases of pulmonary arterio-venous fistula, provides the stimulus for the development of collateral anastomoses between bronchial and pulmonary arteries, then one might expect the anoxemic effects to be overcome in those cases with small fistulas; thus, small degrees of arterial anoxemia might occur, as in case A.B. of the present series. In cases with large fistulas, on the other hand, the collateral flow would probably be insufficient to overcome the deficit in resting arterial blood oxygen saturation, unless it reached enormous and probably disabling proportions.

The size of these tumors, as revealed by x-ray, was remarkably similar in cases H.C., A.B., and S.K.; patient D.K. had a fistula of equal size in both lower lobes of the lung. Although slightly greater variation was found in the excised specimens, there was a conspicuous lack of correlation between the size of the tumor and the volume of blood shunted from artery to vein. Aside from purely anatomical considerations, some of the inconsistency between the size of the fistula and the volume of the shunt may have been due to an additional shunt between the vascular channels of the tumor and the bronchial venous system.* Such a shunt would not be detected by the methods employed here, and, if aerated pulmonary capillary blood were draining into the bronchial venous system, it might invalidate to some extent, the use of the Fick principle as a measure of systemic blood flow. It would not, however, invalidate the inclusion of the right-to-left pulmonary artery to pulmonary vein shunt as a fraction of the flow through the pulmonic valve.

Summary

1. Physiological observations of four cases of pulmonary arterio-venous fistula are reported.

* Under normal circumstances, the drainage through bronchial veins is so slight that it is of little physiological significance.[14, 20]

2. In this group the fraction of total blood flow shunted from pulmonary artery to vein through the fistula varied widely and was not related to the apparent size of the tumor.

3. The expected arterial anoxemia was found to be slightly ameliorated by the presence of a collateral circulation between bronchial and pulmonary arteries in the one case where such an estimate was attempted.

4. The possible rôle of the bronchial veins as an additional shunting mechanism was discussed. No attempt to estimate the volume of bronchial vein flow was made.

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THE PREREQUISITES OF GOOD TEACHING*

ERNEST SACHS

There is an expression that I have encountered repeatedly in talking with some of my medical school colleagues which, I have no doubt, is also used in undergraduate colleges, namely: "spoon feeding." It is a term that is used with disdain, and anyone found guilty of indulging in this practice seems to have damned himself. Apparently, he deserves to be thrown out of the category of those who should be entrusted with the guidance and instruction of youth!

I cannot remember that I have ever heard a definition of spoon feeding. Hence, I am not certain that I know what is meant by it, but I believe that imparting knowledge in a palatable form constitutes this despicable habit. If I am correct in assuming that this is what is meant, I have no hesitation in saying that I always have been, and still am, a firm believer in the baleful practice.

To my mind, imparting knowledge in a palatable, interesting way is the essence of good teaching. It is all very well to say, "Let the student take it or leave it. If he is good, he will take it; if not, *we* don't want him. Let him follow a trade." Yet, what about the student who has not yet been stimulated? One who might be very good if some gifted teacher had deliberately tried to heighten his interest, arouse his curiosity, and stimulate his imagination? Might not such a student respond in an amazing way and show unusual promise? It has happened. For it is not possible to gauge the ability of human beings at a glance. We all respond to different stimuli, different methods of presentation. Now, is not this the real function of a teacher? To find out by what method or methods the greatest development of his students may be brought about? Yet when teaching is discussed at gatherings, far more consideration is given to content than to method. It is my firm and considered belief, however, that content should be taken for granted, and the emphasis placed upon the various methods of presentation.

1. *Mastery of subject.* To me it seems a fundamental principle—axiomatic—that everyone who attempts to instruct others should be a master of his subject. Why else should he have been called upon to teach it? Indeed, he should be so full of it that, except to keep his material up to date, he need not prepare his exercises from day to day. If we accept this premise, then the instructor (I use the term as meaning one who imparts knowledge irrespective of his academic title) has time to consider how best and most effectively he may present this material at his next exercise. If he

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*This lecture was delivered before the faculty of Drury College, Springfield, Missouri in 1944.

does not do this, and relies only upon his own knowledge to make the lesson interesting, it may, and very often has been, deadly dull. I recall an incident which occurred in 1916 in a Greek class. The students were reading in Xenophon the passage about the crossing of the Hellespont. It was dry, lifeless, and so monotonous that the visitor, who told me the story, was himself almost overcome with sleep. At the end of the exercise, he said to the instructor: "Don't you think the students would have been interested to know that the Hellespont is the place where the present Gallipoli campaign is being waged?" This had not occurred to the instructor. He was an excellent Greek scholar, but he was so intent upon the text——and I daresay the literal translation—that he lost this golden opportunity to vitalize a subject that was undoubtedly a bit dull.

About forty years ago when the late President Eliot of Harvard was liberalizing the college course he proposed to drop Greek as a required course for the degree of Bachelor of Arts. Much disturbed, a member of the Greek department came to him and said: " But if Greek is no longer *required*, we shall not have any students," to which President Eliot replied: "If you cannot make your course vital enough to attract students, then really you should not have any."

Here is another example of a man who was a master of his subject, but who failed as a teacher. Some years ago, a group of busy women, all college graduates, decided to make time to keep up certain intellectual pursuits, so they signed up for a Saturday morning extension course given by a great English scholar. This professor, unfortunately, did not like to give extension courses. He felt that all of the women who elected them were at different stages of preparation, and were not serious anyway, so why try to do anything with them? With this attitude of mind, he told them to buy a certain book, gave some assignments, then in class had these read aloud while he looked out of the window unutterably bored. Not a word of comment, no outpouring of the great fund of knowledge that he actually had, nothing for the students to remember but a bored teacher who looked out of the window. Needless to say, the serious students soon dropped the course. He had enthusiasm for study and for writing, but none for teaching. It was unfortunate that this man was obliged to teach instead of devoting his time to the work he did so very well.

2. *Mastery of methods of teaching.* Theoretically, of course, a student should be so interested in his work that he needs no accessory means to stimulate him, yet do we not all remember classes like the Greek exercise in which the teacher lacked life, and the English one in which he lacked interest in his students and enthusiasm for teaching? On the other hand, do not other courses stand out in our minds because certain facts were so picturesquely presented that they have seemed part of our own experience ever since? The late Professor Shaler, head of the Geology Department at Harvard, certainly had the gift of arousing students' interest in his subject.

He was so graphic in his descriptions of places he had seen and studied, and so vivid were they still in his memory, that these same pictures were indelibly impressed upon his students. In his hands the record of the rocks was a living, exciting thing, and it was entirely due to descriptions he gave while I was taking his course that I felt impelled, thirty-five years later, to visit that astonishing natural wonder in the northernmost tip of Ireland, the Giant's Causeway. While looking at that amazing formation, I must admit that I paid silent tribute to a great teacher.

While taking attendance and giving grades are the usual, crude methods of stimulating students to attend classes, how much better to make the course so interesting and vital that they *want* to take it! This matter of making a course interesting, however, brings up a question about which I have heard some debate: that in teaching a subject, extraneous matter should not be introduced, that physics should be taught as pure physics with no relation to allied subjects, and physiology as pure physiology with no reference to its medical connotation. For example, one of the problems that concerns me in taking care of patients with head injuries is the effect of the injury upon blood pressure, pulse rate, and respiration. My students must learn about these things, but the physiologist teaches them on a rabbit. It is quite true that they can be taught on a rabbit, but I maintain that the importance of the observation would be much more vividly brought home to the student if he saw these conditions in a man who had been run over by an automobile and had a severe head injury.

When a student in chemistry is studying oxygen and helium, does it not add greatly to his interest in the subject when he hears or sees what a vitally important part oxygen plays in high altitude flying, or helium in protecting divers from "the bends"?

I believe that the mathematicians, earlier than any other teachers, realized the need of introducing practical examples in their work. It was about forty to fifty years ago that the teaching of mathematics was completely changed. Certainly an entirely new type of textbook came into use at about that time, in which all sorts of mathematical problems were illustrated by practical examples, such as finding the height of mountains or church steeples rather than giving merely abstract figures.

Since then, illustrative material has come into use in teaching various subjects and is unquestionably helpful in arousing the interest of the students. There is no one technique, however, that is applicable at all times, or to all subjects. Different teachers use methods of their own devising. For instance, the great Professor Neusser of Vienna used to bring in a patient and point out some abnormality, such as jaundice, then, completely disregarding the patient and drawing upon his vast experience for as long as an hour, he would discuss the various conditions in which jaundice might occur. The next day he would bring the patient back and in the light of what he had discussed would work out the diagnosis before his students.

In medical education, with which I am particularly familiar, great changes have occurred in the past thirty years. I believe one may date the change with the founding of Johns Hopkins, when that extraordinarily keen judge of men, Daniel Coit Gilman, selected for his faculty the outstanding group of *teachers* that made the medical school the leading school of its day. The great change I refer to consisted in discarding, to a large extent, the formal lecture for a more practical exercise in which the student no longer was on the receiving end only, but took an active part in the exercise. This was the development of the modern clinic in which disease processes were demonstrated either on the living person or on the organs obtained from a patient who had died of the disease. But even these exercises can be dull and uninteresting if the teacher has not learned that much neglected device, the use of the question.

Professor DeGarmo, in his book entitled *Interest and Education,* has a fascinating chapter on "The Art of Questioning." "To question well," he says, "is to teach well. In the skillful use of the question, more than anything else, lies the fine art of teaching." He then goes on to analyze the different classes of questions and gives countless examples of the correct and incorrect method of asking them. Every question should be definite, clear, and calculated to stimulate thought. A properly framed question tests the pupil's state of mind; it may lead him to venture farther along ways that have been suggested, and may even prompt him to investigate entirely new paths for himself. What greater reward could a teacher ask?

It seems to me very poor teaching to ask set questions for which there are stereotyped answers. Our guide in questioning should always be the student's response, and if this is not taken advantage of, the clue for the next question is deliberately thrown away. I realize that this may be more difficult of application in some subjects than in others, but a timely question may illuminate an entire problem.

Frick, one of the great pedagogues of Germany, made the following summary: "A properly devised questioning process must lead systematically from a definite body of connected information through a definitely connected chain of thought to a definite conclusion. The starting point must be assured, clear of misunderstandings; the progress must be logical, without break of continuity or treacherous overlapping of statements; the pupils' conclusion must be precise, must grow of necessity out of the data offered, must not permit of alternative results."

Unfortunately, the ability to ask questions effectively is a greatly neglected art. Perhaps it has been forgotten, perhaps it is not prized as it should be or recognized as of prime importance. In any case, questioning in such a way that the answer will throw light on the subject and make the presentation more vivid *is* a true art that requires much practice.

Charles Downer Hazen, Professor of History at Smith and later at Columbia, was a past master of this art. He wrote a superb book entitled

Europe since 1815 which was used as a textbook by his students. On one occasion he began the exercise with the question: "Who was the greater man—Bismarck or Cavour?" With this basic question, he developed, in the course of an hour, the history of the entire period. During that time he may have called upon fifty students. They had to say what they wanted to say quickly or he called on others, but he did no talking himself. Another time he asked: "Why is land so precious in England?" The answer was "Because of the law of primogeniture which concentrates the land in the hands of a few," but the hour was over and the gong was ringing before this conclusion was reached by the excited class. Dr. Hazen might have asked many questions which would have brought out the facts, but these two were such a challenge to the students and so stimulated them that they have never forgotten that course. It was so interesting, so exciting, that the students felt they had to be on their toes in *that* class, whatever else they neglected.

One of the greatest compliments I ever received from a student was when he said: "You know, Dr. Sachs, I can never tell from your question what the answer should be." In other words, at least I so interpreted it, my questions had made him think and reason. And should not this be the end and aim of teaching?

We all, I imagine, have had the experience of coming out of a classroom and thinking, "That was a poor performance. The students were not *with* me," or at other times, "Today *I really got my ideas across.*" When I have come to anaylze the poor exercise, I have not infrequently found that the trouble lay in my having asked some non-productive questions, those which had not stimulated the student to think, or perhaps had led him off into a path that terminated in a blind alley. I believe that even experienced teachers have their off days, but far less frequently than the inexperienced ones.

Yet, though proper questioning is the most important part of teaching, there is a definite field for exposition, narration, and description which should be used in part in formal lectures. In school or college teaching, however, that type of lecture should be discarded more and more, giving place to a constant give and take between instructor and students. When there must be lecturing, it will be found that exposition, narration, and description will be greatly helped by dramatization. In fact, the most effective teacher makes use, at times, of some of the tricks of the dramatist and certainly none is more telling than suspense and surprise.

In recent years a method of questioning has been developed which I, personally, think is very unfortunate and stultifying. Many short questions are asked, as many as a hundred, in each of which a word is omitted which must be filled in, or perhaps, several words are used and the student has to cross out the incorrect ones. Also statements are made which differ widely, and all the student has to do is mark them false or true.

The only purpose that I can see for this method is that the papers can be graded accurately. Certainly it gives no idea of the students' mental

processes. Furthermore, these tests are often so long that speed becomes one of the prime factors in the test. Speed, in most intelligence tests, is supposed to be of vital importance, but *I* do not believe that speed is any true test of intelligence. Some of the ablest scholars have been very deliberate and have arrived at conclusions slowly. Had they taken the modern intelligence test, I fear that they might have been given a very low—and therefore absolutely incorrect—rating. Some years ago I had an opportunity of discussing the matter of these tests with the late Professor Woodbridge of Columbia, and I wish I could repeat to you his caustic criticism of this method. There was no question where that great philosopher stood on the matter of so-called intelligence tests.

3. *Improvement of methods.* I should like now to consider what can be done to improve teaching in this country, what can or should be done to help young teachers who are just beginning. I venture to suggest a solution which we have not followed to any great extent, but which I am confident would help to correct many of our difficulties. It is a method that was used in preparing teachers in Germany when that country still prized culture. Just because a man knows his subject does not make him a teacher, as we have seen. In this country, when a young man is appointed to a position, he is turned loose on the student body with nobody to guide or supervise his work or to help him. Why? Because there exists a very general feeling that it is embarrassing to a young man to have the head of his department sit in on his classes to observe what he is doing. It is supposed to indicate lack of confidence in his ability. So he is left to feel his way and learn for himself how to teach. Meanwhile the students suffer.

Several years ago, a young surgeon, who had been turned loose in this way, came up to me fully five years after he had been instructing students and said: "I wonder whether you would give me some points about teaching. I have just lately come to realize that I ought to know something about it. There is a kind of technique, isn't there?" The commendable thing about this young man was that he had finally come to realize that there was something to this "business of teaching," but he had not grasped it sufficiently, for he thought it required just a few suggestions which could be given in a few minutes' time. Why does this situation exist, and why do we permit it to go on?

The question, as applied to young men, has just been answered. When older men are appointed to a college or university position, the question primarily asked is: "What has he published?" and usually "How much?" Quantity, in many instances, seems more important than quality. Very, very seldom is the question asked: "Can he teach?"

Some time ago, I was consulted about the selection of the head of an important department and was given a list of names, after each of which was a list of his publications. Nobody inquired, or knew, or seemed to care about the ability of these men as teachers. As a matter of fact, the man who

was finally selected was no teacher, was not interested in teaching, even disliked it, and has done very little since he was appointed. He is a research man. I do not undervalue research and firmly believe that the head of a department and his assistants should be productive, carrying on investigation as far as time permits, but their prime function is to teach. Furthermore, I believe, as did my great teacher, William Osler, that the introduction of a subject should be taught by the head of the department because it is of vital importance that the young people be properly introduced to a new subject. This, presumably, is best done by the one in charge of the department.

Referring again to the training of young men—why should not the head of a department tell a new, inexperienced man that he will attend a number of his classes with the idea of helping him in his work? It certainly is done in business. No perfectly green accountant, clerk, salesman, or even executive would be allowed to go his own, sweet way without supervision. Why should colleges and universities be so much less efficient? Then, too, it would help the whole situation greatly if, before appointing a man to teach, someone could hear what he could do. But if a young man is appointed who has never taught before, he certainly needs and deserves some help and guidance.

It was my misfortune that when I was first appointed, I had never done any teaching and I had to learn by myself. According to the usual rule, my chief did not believe in visiting my classes, and I had to evolve my own technique. Such success as I may have had, I attribute to three factors. First, I was intensely interested in and enthusiastic about teaching, and after some thirty years of it, I think I may say that I love it and always find it a challenge; secondly, I know I was helped by the fact that I had a father who, in his line, was known as a great teacher, and he both instructed and inspired me; in the third place, I had had the good fortune to have studied under great men—great teachers—both here and abroad. Of these three factors, the most important, I feel, was interest and enthusiasm. If a man is not keen about his job, irrespective of what it may be, he will not do it well.

An institution, in appointing a man, must decide which it prefers—teaching or research. Though these two are not antagonistic or incompatible, it is most unusual, in my experience, to find in one individual both an outstanding teacher and an outstanding investigator. Entirely different qualities are required. Which shall it be, and how shall the institution deal with this serious dilemma? It is particularly difficult for a small institution with limited means to have both types of men, but it seems to me that each man should be assigned to the job that he can do best. Let those who are particularly adapted for and interested in research do that, and let the teachers teach. In so doing, however, we should not make the mistake of placing research on a higher pedestal than teaching, as is frequently done. It would be ideal if there could be two heads of a department, one in charge

of teaching and the other in charge of research. Yet most institutions are not in a financial position to do this. They might, however, accomplish the same result by having different men give the same course in different years, or better still, by a system of interchange with other institutions. We have developed a system of inbreeding in our colleges, which is akin to nationalism and isolationism in political life. Colleges believe it is a reflection on their efficiency if they are not entirely self-contained, self-sufficient. Yet it is obvious that most institutions cannot be equally strong in all departments. Would it not make for *greater* efficiency if a more extensive system of exchange professors were developed? And if it were also made possible for students to go to different institutions to work with men who are outstanding in their field? Then the students would have the added opportunity of imbibing some of the atmosphere and spirit of the university they were visiting. At several of the women's colleges, the junior year was taken in France or Spain or Germany in the pre-Hitler days, but the same principle could be carried out within the country. This interchange lapsed for a time after the war but now it has been reëstablished and there is more activity than ever before. The Fulbright fellowships have contributed a great deal to make this possible.

In Germany, when a man received the title of Docent, he was privileged to offer courses in his field and receive fees from each student. If his course was interesting, it was well attended and he received considerable compensation; if it was dull, he had few or no students. This system existed in the small universities in small cities such as Freiberg and Giessen, which were no larger than Springfield or Jefferson City. In the German system, students went to study under certain men, not in certain institutions. An application of this system might, I believe, be of great value in this country. For example, there died recently in Galveston a physiological chemist by the name of Bodansky who wrote such a superb book in his field that it was the required textbook at most of the leading medical schools in this country. He was also a stimulating, inspiring teacher. Imagine what a wonderful thing it would have been for the University of Texas, and for Bodansky himself if, when this course was given, students had flocked to him from Harvard, California, Chicago, Virginia, etc.

Such interchanges may seem insurmountable at first glance because of the financial difficulties involved, but here is where our great foundations could step in and do most constructive work. They are always ready to enter new fields which give promise of yielding a valuable return. I know of no more fundamental and promising field they could embark upon than this.

At first, this would have to be in the nature of an experiment. If six or a dozen colleges would agree to unite forces, the feasibility of such a scheme could be demonstrated, and from this experiment, a more comprehensive

plan could be evolved which gradually might include all of the leading institutions in this country.

We are much concerned, these days, about the future of our youth. They will need help in many directions. After having made the great sacrifices they made during the war, surely they deserve the best. What more fundamental contribution could we make than to give them the best training in the most effective way possible?

In conclusion, I should like to sum up what seem to me to be the prerequisites of good teaching:

1. Know your subject thoroughly.
2. Be enthusiastic about teaching.
3. Be sincerely interested in students.
4. In exposition, be simple, logical, lucid.
5. In description, make the student see what you see.
6. In narration, arouse his curiosity.
7. Dramatize whenever possible, making use of suspense and surprise.
8. Link your subject matter with the everyday experience of your students.
9. Use illustrations and illustrative material whenever possible.
10. Make the student take an active part in every exercise.
11. Use his response as a guide for the next question.
12. Be adept in the art of questioning.

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THE PARTIAL PURIFICATION OF THE LETHAL TOXIN OF *CLOSTRIDIUM HISTOLYTICUM**

HARRY E. BOWEN**

Although several bacterial toxins have been prepared in highly purified form (c.f. Pappenheimer,[6] Lamanna *et al.*,[4] and Pillemer[8]), comparatively little effort has been made in this direction with the toxins of the gas gangrene organism *C. histolyticum*. Weinberg and Seguin[11] demonstrated that it could be precipitated with ammonium sulfate, and made the observation that ethanol destroys the toxic activity. Stewart[9] prepared stable toxin preparations by precipitating culture filtrates with saturated ammonium sulfate, followed by drying over phosphorous pentoxide. Aside from these reports, there is no further information, to the author's knowledge, concerning possible means of concentrating the toxin.

This study was undertaken in an attempt to determine which of the usual methods of separating proteins could be applied to the lethal toxin of *C. histolyticum* and attempts a comparison of the products obtained by the various methods.

Materials and Methods

Toxin production. The liver digest medium employed and the conditions of growth have been described in another communication.[2] The organism used was Spray's *C. histolyticum* strain H 32.

Assay of toxicity. The graphical method of Litchfield and Fertig[5] was used to determine lethal toxicity which is expressed as the 50 per cent endpoint (ED_{50}) and the slope (b) of the dosage-effect line. Normal 15-20 g. mice of various inbred strains from the laboratory of Dr. L. C. Strong were used as the test animals. Each assay was performed using five groups (i.e. dosage levels) of ten animals each, the animals being assigned to a particular group by the use of a table of random numbers. The mice were injected intravenously with 0.2 ml. of the appropriate freshly prepared buffered saline dilution of toxin. Final recordings of death were made at 72 hours.

Protein nitrogen determinations. Fractions to be analyzed for protein nitrogen (N) were first dialyzed through cellophane against distilled water at 5° C. for at least 24 hours or until free from ammonium ions as indicated by a negative reaction to Nessler's reagent (sensitive to 0.001 mg. ammonium nitrogen per ml.). Kjeldahl nitrogen determinations, in duplicate, were made on the non-dialyzable portion according to the procedure of Kabat and Mayer.[3] The values of N recorded represent the mean of the duplicate determinations corrected for the dilution involved in the dialyzing procedure.

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Concentration of the Toxin

Crude dried toxin. A modification of Stewart's[9] method was used to prepare a large amount of stable crude toxin as a starting material for further attempts at purification. Ten- to thirteen-hour cultures were filtered first through filter paper pulp, then a Mandler candle. The toxic filtrate was then precipitated by bringing to saturation with ammonium sulfate powder (750 gm. per liter). The brown precipitate floated to the surface where it was easily collected. The precipitate was allowed to drain in a Buchner funnel, and finally dried *in vacuo* over $CaCl_2$. In this manner, a total of 80 liters of filtrate were worked up, yielding 375 g. of dried crude toxin and representing approximately 87 per cent recovery of the original toxic activity of the culture filtrate.

Further concentration of this crude toxin was attempted by three fundamentally different techniques: (i) salting out with ammonium sulfate; (ii) ethanol fractionation in the cold; and (iii) precipitating with the chloride salt of the heavy metal cadmium. Extensive preliminary experiments were conducted to determine the ideal conditions for each of these three techniques. On the basis of these experiments, several methods of concentration offering promise were developed. They are described below.

Fraction A. "Crude dried toxin" is suspended, at room temperature, in distilled water (4 g. per 100 ml.). After dissolving as completely as possible by stirring with a glass rod, the suspension is centrifuged (2000 R.P.M. for 30 minutes). The clear, dark supernate is decanted, leaving behind a small amount of inactive, insoluble material which is discarded. The supernate, which usually has a pH of about 6.0 is then adjusted to pH 7.0 with 10 per cent NaOH. This solution, designated Fraction A, is, then, simply a neutral solution of crude toxin freed of insoluble matter.

Fraction AI. Fraction A is brought to 20 per cent saturation with ammonium sulfate (SAS), and the resulting suspension centrifuged at 2000 R.P.M. for 30 minutes. The supernate is decanted (leaving behind a small amount of inactive precipitate to be discarded) and further treated to 60 per cent SAS. This suspension is then centrifuged in the usual manner and the clear supernate discarded. The precipitate is suspended in $\frac{1}{8}$ the original volume of M/15 phosphate buffer, pH 7.4. After centrifuging, the supernate, designated Fraction AI, is collected.

Fraction AII. Fraction AII is prepared from AI by bringing AI to $\frac{1}{2}$ the original volume (of Fraction A) with phosphate buffer and precipitating with 60 per cent SAS. After centrifuging, the precipitate is suspended in $\frac{1}{16}$ volume (of Fraction A) of phosphate buffer. The supernate resulting from centrifuging this suspension is designated Fraction AII.

Fraction CI. Fraction A is precipitated with 1/25 volume of 5 per cent cadmium chloride solution at room temperature. Following centrifugation, the clear, dark supernate is discarded and the precipitate suspended in $\frac{2}{5}$

volumes (of Fraction A) of distilled water. After centrifugation, the supernate is discarded and the precipitate suspended in ⅛ volume of phosphate buffer giving Fraction CI.

Fraction EI. Fraction A and ¼ volume of 95 per cent ethanol are chilled to slightly below 5° C. in an ice bath. The alcohol is added to the toxin solution slowly and with constant stirring, taking care never to allow the temperature of the mixture to rise above 5° C. (Extreme care must be taken. Since considerable heat is generated when ethanol and water are mixed, the temperature can rise quite rapidly to above 5° C.) The mixture is centrifuged in a refrigerated centrifuge at 5° C., and the supernate decanted, leaving behind a small amount of non-toxic precipitate.

This supernate is precipitated with an additional 1¾ volumes of ethanol, maintaining the temperature below 5° C. This mixture is also centrifuged, and the clear, dark supernate discarded. The centrifuge bottle containing the precipitate is allowed to drain 30 minutes in the cold in order to remove as much alcohol as possible. Finally the precipitate is taken up in ½ volume (of Fraction A) in phosphate buffer, and centrifuged at room temperature to yield the clear supernate designated Fraction EI.

Fraction EII. Fraction EI (which should be at pH of 7.3) is chilled to below 5° C. and precipitated by the addition of ½ volume of 95 per cent ethanol which has also been previously chilled. After centrifuging at 5° C., the supernate is discarded, and the precipitate allowed to drain 30 minutes. Finally, the precipitate is suspended in ⅛ volume (of Fraction A) of phosphate buffer, centrifuged at room temperature, and the supernate, Fraction EII, is collected.

Comparison of the Different Fractions

Several lots of concentrated toxin were prepared by each of the methods described. On each lot determinations of protein (i.e., non-dialyzable) N, ED_{50}, and slope of the dosage-effect line were made. From these data, the "purification factor" and "per cent recovery" were determined. The purification factor is simply the ratio of the ED_{50} in mg. protein N of the crude filtrate over the corresponding value for the purified product. This value reflects the ability of the fractionation procedure to eliminate contaminating proteins. The per cent recovery is the ratio ($\times$ 100) of the total ED_{50} in a purified lot of toxin over the total ED_{50} of the crude filtrate from which it was derived. As such, it is a measure of the efficiency of the fractionation procedure, indicating how much of the initial toxic activity is carried over in the final product.

The results obtained on a number of purified lots of toxin are given in Table 1. The data are self-explanatory. Their significance and interpretation are taken up in the discussion.

Discussion

The usefulness of any method designed to purify toxins is based upon its ability to separate the toxin from non-toxic material and to effect as little loss of the toxin as possible (either through actual destruction or through failure to effect a clear separation). Therefore, in evaluating any such methods, the most important characteristics to consider are the "per cent recovery" and the "purification factor," the former measuring the loss of

Table 1

Comparative Properties of the Various Toxic Fractions

Fraction	*Reduction in volume*	*Protein N per ml. (mg.)*	*ED_{46} in* mg. N ($\times 10^{-4}$)*	*Purification factor*	*Per cent recovery*	*Slope* of dosage effect*
Filtrate	1	1.31	52.0	1	100	.....
		1.67	8.3 ± 0.4	6.3	93	13.47 ± 5.3
A	8.5	1.69	9.6 ± 0.4	5.5	83	10.67 ± 7.0
		1.70	8.3 ± 0.5	6.3	96	9.48 ± 3.8
		5.91	10.5 ± 1.2	5.0	33	5.22 ± 1.6
AI	68	6.10	9.3 ± 1.2	5.6	38	6.56 ± 3.7
		7.50	6.6 ± 0.8	7.9	33	6.56 ± 1.9
AII	136	7.73	8.4 ± 0.7	6.2	27	7.31 ± 2.0
		0.601	0.66 ± 0.05	79	53	8.53 ± 2.0
CI	68	0.580	0.67 ± 0.04	79	51	10.63 ± 6.9
		0.069	0.36 ± 0.03	144	45	8.83 ± 1.3
EI	17	0.071	0.34 ± 0.02	153	49	9.85 ± 1.9
		0.074	0.34 ± 0.02	153	51	11.13 ± 7.7
		0.114	0.41 ± 0.05	127	16	5.82 ± 1.5
EII	68	0.099	0.32 ± 0.04	163	15	7.76 ± 1.5
		0.101	0.34 ± 0.04	153	14	6.92 ± 2.7

* Plus or minus the standard error.

toxic activity during treatment and the latter the degree to which the separation from contaminating material has been accomplished. An examination of the data in Table 1 indicates that a fair separation is accomplished by a single precipitation with $(NH_4)_2SO_4$ (Fraction A), and that the recovery of toxin is very good. Further fractionation with ammonium sulfate under the conditions described (Fractions AI and AII) is without value, since it fails to increase the purity of the toxin, and, indeed, considerably reduces the recovery. The use of cadmium chloride solutions to concentrate the toxin brings about a marked increase in purity while allowing roughly 50 per cent total recovery of toxin. By far the best results were

obtained with ethanol fractionation in the cold (Fraction EI). Here the recovery was almost the same as with cadmium chloride precipitations, while the purity of the preparation showed an appreciable improvement. The separation effected by this method can best be appreciated by a consideration of the amount of contaminating material removed. From the data, it can be seen that 1 ml. of filtrate contains 1.31 mg. N, while 1 ml. of Fraction EI, which is derived from 17 ml. of filtrate contains about 0.07 mg. N. Thus, $\frac{(17)\ (1.31) - (0.07)\ (100)}{(17)\ (1.31)}$ or roughly 99.7 per cent of the original protein has been eliminated while 50 per cent of the original toxic activity has been retained. Under these experimental conditions, a second fractionation with ethanol (EII) does not increase the purity of the toxin and brings about a marked reduction in recovery.

TABLE 2

ANALYSIS OF SLOPES OF DOSAGE-EFFECT LINES BY CHI-SQUARES

Fraction	*Individual slopes*	*chi-square*	*Degrees of freedom*	*P*
A	13.47, 10.67, 9.48	0.38	2	0.8—0.9
AI	5.22, 6.56	0.11	1	0.7—0.8
AII	6.56, 7.31	0.075	1	0.7—0.8
CI	8.53, 10.63	0.080	1	0.7—0.8
EI	8.83, 9.85, 11.13	0.26	2	0.8—0.9
EII	5.82, 7.76, 6.92	0.88	2	0.5—0.7
All Fractions	.....	6.73	14	0.9—0.95

The slope of the dosage-effect line is a characteristic of any toxic substance. Therefore, if, in analyzing the dosage response lines of two toxic preparations, significant differences in their slopes are observed, one would conclude that each preparation contains a different toxin (or perhaps several toxic substances in different proportions). If the slopes did not differ, one could conclude that either the two preparations contained identical toxins, or, less likely, different toxins with dosage-response lines which just happened to be the same. An examination of the data in Table 1 reveals that the slopes vary from 13.47 to 5.22. However, due to the limited number of animals used, few points went into the determinations of these slopes, with the result that the standard errors (SE_b) are quite large. Analyzing these data by the chi-square method of Fertig,[1] the results given in Table 2 are obtained. These findings indicate no significant differences between the slopes of the individual preparations. On the basis of this analysis, then, the hypothesis that the true dosage-response curves of each of the various fractions would give identical slopes is quite tenable. However, the experi-

mentally determined slopes are actually so different by direct comparison that one hesitates to make too strong a statement. The difficulty here, of course, arises from the inherent inaccuracy of the experimentally determined slopes and the consequent large errors. The satisfactory answer would be obtained by toxicity determinations involving more mice. Unfortunately, the numbers required for such an undertaking were not available. It may be concluded, keeping in mind the above reservations, that it is likely that all fractions (by virtue of this probable identity of slope) may contain one and the same lethal toxin. In view of the widely different methods of fractionation employed, this suggests that strain H 32 produces a single lethal toxin.

C. histolyticum is often considered to be only weakly toxigenic, and, indeed, has been described as non-toxigenic. It is interesting, therefore, to compare the toxicity of the partially purified preparation described here with other purified bacterial toxins, for example:

Toxin	*M.L.D. per kg. mouse per mg. toxin*
Botulinus, Type A	620,000
Tetanus	200,000
C. perfringens, alpha toxin	200
C. histolyticum, fraction EI	85
Diphtheria	3.5

These figures, taken from Pappenheimer[7] and Van Heyningen,[10] place *C. histolyticum* toxin in a class with diphtheria and *C. perfringens* alpha toxin as far as potency for mice is concerned.

Summary

Several methods of concentrating the lethal toxin of *C. histolyticum* are presented. Of these methods, fractionation with ethanol in the cold offers the most satisfactory method from the point of view of total recovery of toxin and degree of purity achieved. The best preparations obtained by ethanol fractionation show a toxicity for mice intermediate between that of purified *C. perfringens* alpha toxin and diphtheria toxin. Statistical analysis of the dosage-response curves suggests that the strain of *C. histolyticum* used produces a single lethal toxin under these experimental conditions.

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A COMPARISON OF THE LETHAL AND HEMOLYTIC TOXINS OF *CLOSTRIDIUM HISTOLYTICUM**

HARRY E. BOWEN**

Weinberg and Seguin,[7] shortly after first isolating *Clostridium histolyticum*, demonstrated that broth filtrates are lethal for laboratory animals when injected intravenously. These same preparations injected intramuscularly into the thighs of experimental animals resulted in rapidly progressive histolytic lesions. (The pathology of these lesions has been described in detail by Pasternack and Bengtson[8]). Later, Mita[1] reported the production of a heat labile hemolysin in liver broth culture filtrates.

Stewart,[9] in a detailed investigation of the production of toxin by *C. histolyticum*, found that potent toxins could be produced in a peptone-meat infusion broth at pH 7.6. Toxic activity reached a maximum after incubation for 13 to 15 hours at 37° C., and then fell off sharply. She also showed that the addition of 2 per cent glucose to media containing Parke-Davis peptone markedly increased the yield of toxin and resulted in the production of a potent hemolysin. This stimulating effect was attributed to "the reducing action of the glucose" for the following reasons: (i) other reducing sugars had the same effect while non-reducing sugars did not; (ii) glucose is not metabolized by *C. histolyticum* and its presence fails to result in increased growth of the organism; and (iii) the presence of glucose in Parke-Davis peptone media was shown to accelerate the reduction (as indicated by reduction of various dyes) of the medium by *C. histolyticum*. Furthermore, she found that media containing Witte peptone, which is rich in reducing sulfhydryl groups, would bring about the production of potent lethal and hemolytic toxins whether or not glucose was added.

Sigler[5] confirmed Stewart's findings regarding the production of toxin and hemolysin. On the basis of the similar conditions required for the production of lethal toxin and hemolysin, he suggested that the two activities are due to a single substance. It is the purpose of this investigation to examine his suggestion in greater detail. Should they prove to be identical, hemolytic activity might provide a convenient *in vitro* method of assaying for lethal toxicity as is now the case with the alpha toxin of *C. perfringens*.

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Materials and Methods

Ten strains of *C. histolyticum* from various sources were examined in an effort to find a morphologically and physiologically typical one which was capable of producing filtrates highly toxic for mice upon intravenous injection. Dr. R. S. Spray's strain H32, which was received from the National Institutes of Health, was selected as the organism best meeting these requirements and was used throughout this study.

The basic formula for the toxin medium and a supply of papain digest of liver cake, its primary ingredient, were very kindly supplied by the Lederle Laboratories at Pearl River, New York. The medium, as modified by us, has the following formula:

Dried papain digest of liver cake	40 g.
Glucose	20 g.
$Na_2HPO_4 \cdot H_2O$	5.7 g.
KH_2PO_4	0.48 g.
$MgSO_4 \cdot 7H_2O$	0.02 g.
Welch's Vitamin solution*	1.0 ml.
Distilled water	to 1 liter

The pH was adjusted to 7.5 with NaOH before sterilizing at 15 pounds pressure for 20 minutes.

Medium was dispensed in one-liter flasks in approximately 750 ml. amounts, sterilized, and then rapidly cooled to 37° C. in a water bath. It was inoculated immediately with 7.5 ml. of a 24-hour broth culture of strain H32 and incubated at 37° C. Sterile filtrates were prepared by filtering the culture first through filter paper pulp and next through a Mandler candle. Since culture filtrates are generally weak in hemolytic activity, broth supernates (2000 RPM for 30 minutes) were also used in the investigation of the properties of the hemolysin and of the lethal toxin.

Toxicity was determined by the LD_{50} method of Reed and Muench[4] using albino mice of 20-25 g. weight. The animals were injected intravenously with 0.2 ml. of the appropriate saline dilutions of broth filtrates or supernates. Final recordings of death were taken after 72 hours, since preliminary experiments demonstrated that most of the deaths occurred within the first 48 hours, with none after 72 hours.

Rabbit red blood cells (RBC) prepared fresh daily as a 2 per cent suspension in saline were used in testing hemolysis. One ml. each of RBC suspension and broth filtrates or supernates (in serial two-fold dilutions) were mixed in Wassermann tubes and incubated in a 37° C. water bath for two hours. The tubes were read at this time and again after storage overnight at 4° C. The degree of hemolysis was determined macroscopically and the individual tubes assigned a value of 4, 3, 2, 1, or 0 to represent approximately complete, 75 per cent, 50 per cent, 25 per cent, and no hemolysis respectively. The data are presented as the "sum of degree of hemolysis" (SDH) which represents the total of the individual readings for all dilutions in any one titration.

Experimental

Toxin production in relation to growth. Cultures of strain H32 were grown in freshly autoclaved flasks of broth as already described, and samples were withdrawn at intervals for testing. Turbidity, as determined at 650 mμ. on the Klett-Summerson colorimeter, was used to measure growth.

* Welch's vitamin solution contains pimelic acid (100 mg.), pyridoxine (100 mg.), thiamine hydrochloride (100 mg.), calcium pantothenate (100 mg.), choline (100 mg.), nicotinic acid (100 mg.), and riboflavin (10 mg.) all dissolved in 400 ml. of 50 per cent ethanol. This solution was stored at 4° C. until needed.

The toxic and hemolytic activities of broth supernates were determined in the manner described. A comparison of these three properties (average values from five individual experiments) is shown in Figure 1.

These findings confirm the reports of Stewart[6] and Sigler[5] regarding the early toxin production and subsequent rapid decline in activity. It is also evident that the production of toxin and hemolysin is directly correlated with the logarithmic phase of growth of the organism, a period ending usually after 9-12 hours of incubation. At the conclusion of the logarithmic phase, lethal and hemolytic activities fall off rapidly so that within approximately three hours the LD_{50} has dropped two-fold.

Oxidation. The findings of Stewart[6] on the effect of reduction on hemolysin production, and the fact that both broth supernates and filtrates stored in air in the cold lose their hemolytic activity of *C. histolyticum*, like certain other bacterial hemolysins, may be controlled in part by oxidation and reduction. Preliminary experiments indicated that supernates stored overnight at 4° C. showed no loss in lethal activity but a marked decrease in hemolytic activity. When hemolysin so stored was tested in the presence of a final concentration of 0.1 per cent sodium thioglycollate, however, it again showed its original activity.

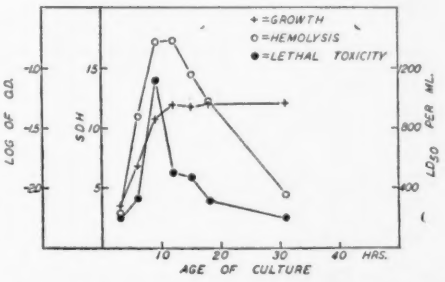

FIG. 1. Comparison of bacterial growth and lethal and hemolytic activities of *C. Histolyticum*.
SDH values are for supernate undiluted and diluted 1:2, 1:4, 1:8, and 1:16.

Aliquots of broth supernates (or filtrates) were stored in various atmospheres at 4° C., and tested at intervals of 1, 3, and 7 days for hemolytic and lethal toxicities. Twenty ml. of a broth supernate preparation were placed in each of four 500 ml. Erlenmeyer flasks, thereby providing a large surface area for a small volume of fluid. The atmosphere in one flask was replaced with oxygen and the flask was tightly stoppered immediately. The second flask was similarly prepared with air, the third with nitrogen, and the fourth with nitrogen after the addition of sodium thioglycollate (0.1 per cent) to the supernate. Testing for hemolytic activity was done by two methods: by simple mixing of hemolysin and RBC as already described (non-reduced), and by adding sodium thioglycollate, to a final concentration of 0.1 per cent, to the mixture of RBC and hemolysin immediately before immersing in the water bath (reduced).

The findings of a typical study with broth supernates (Table 1) show that, even in the most reduced preparations, hemolytic activity is slowly lost, inactivation being most pronounced in an oxygen-laden atmosphere. Only part of the activity lost can be recovered by testing in the presence of a reducing agent. In this property it resembles tetanolysin, which Neill[9] found to be only partially reactivated by reduction. On the basis of this

TABLE 1

ACTIVITY* OF HEMOLYSIN STORED IN VARIOUS ATMOSPHERES AND TESTED IN REDUCED AND NON-REDUCED SYSTEMS

Length of storage	*Oxygen*		*Air*		*Nitrogen*		*Nitrogen; thioglycollate added*	
	Non-Reduced	*Reduced*	*Non-Reduced*	*Reduced*	*Non-Reduced*	*Reduced*	*Non-Reduced*	*Reduced*
0—Control	..	..	18	19	..	..	..	..
1 day	8	8	10	12	11	13	..	18
3 days	3	9	3	11	5	13	..	18
7 days	0	7	1	6	1	9	..	15

* Expressed as SDH for dilutions 1:1, 1:2, 1:4, 1:8, and 1:16.

TABLE 2

THE EFFECT OF SHAKING HEMOLYSIN IN VARIOUS ATMOSPHERES, TESTING IN REDUCED AND NON-REDUCED SYSTEMS*

Test system	*Unshaken control*	*Shaken 1 hour in an atmosphere of:*			
		Oxygen	*Air*	*Nitrogen*	*Nitrogen; thioglycollate added*
Non-reduced	17	3	11	10	..
Reduced	19	3	11	10	13

* Expressed as SDH for dilutions 1:1, 1:2, 1:4, 1:8, and 1:16.

behavior, Neill postulated that tetanolysin was inactivated in two steps, only the first (i.e., oxidative) being reversible.

Concurrent titrations of mouse toxicity showed no change, the preparations retaining a lethal activity of 500 LD_{50} doses per ml. even after seven days' storage in oxygen. In view of these findings, all subsequent determinations of hemolytic activity, unless otherwise indicated, were performed in a reduced system (0.1 per cent thioglycollate added immediately before incubating).

Surface denaturation. The effect of surface denaturation was examined by preparing four flasks as in the previous experiment, and exposing them to one hour's shaking (275 shakes per minute) in a shaking machine. Hemolysin is inactivated by this treatment (Table 2), the effect being most marked

in the presence of oxygen. In contrast to the findings with storage in various atmospheres, inactivation here was not reversible by reduction, even where oxygen apparently played a definite rôle in the inactivation. Lethal toxicity for mice was not altered by this shaking treatment.

Heat and reaction. In broth filtrates and supernates at pH 7.2-7.4 the hemolysin is partially inactivated by 10 minutes' exposure to 45° C., and completed inactivated at 50° C. for 10 minutes. The lethal toxicity is somewhat more stable, being completely inactivated at 55° C., but not at 50° C. for 10 minutes.

Supernates and filtrates adjusted to pH values ranging from 2.0 to 9.0 (using HCl or NaOH) and stored at 5° C. for 24 hours show marked differences in hemolytic and lethal activity when readjusted to neutrality and tested (Figure 2). The lethal activity is stable from pH 9 to pH 6, a more acid environment causing a decrease in activity. The hemolysin, on the other hand, is most stable at pH 6, with activity decreasing in either more alkaline or more acid conditions.

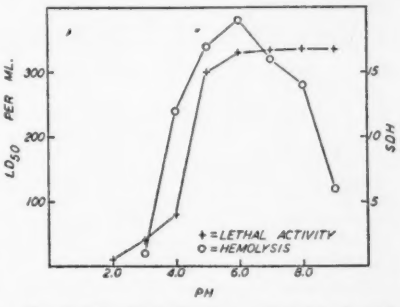

FIG. 2. The effect of pH upon lethal toxin and hemolysin (stored at the indicated pH 24 hours at 5° C.). SDH values are for supernates undiluted and diluted 1:2, 1:4, 1:8, and 1:16.

Adsorption by erythrocytes. The possibility of separating lethal and hemolytic activities by adsorption on RBC was investigated in the following manner. Undiluted broth supernate and 2 per cent RBC were mixed in equal amounts in the cold. The mixture was held at 5° C. for two hours, then centrifuged to remove the RBC before lysis occurred. The resulting supernate was then tested for both lethal and hemolytic activities. The control was simply diluted with an equal amount of saline and incubated with the test mixture at 5° C.

TABLE 3

ACTIVITY OF CULTURE SUPERNATES FOLLOWING EXPOSURE TO RBC

Treatment	*LD_{50} per ml.*	*SDH**
Untreated control	500	16
Adsorbed at 5° C.	500	0
Control at 5° C.	500	16

* For dilutions 1:2, 1:4, 1:8, and 1:16.

The results (Table 3) indicate that the hemolysin can be adsorbed by RBC leaving the lethal toxin with unchanged activity in the supernate.

Effect of proteolytic enzymes. Enzyme solutions were prepared by filtering 0.4 per cent suspensions of each enzyme (Pepsin, Lilly USP; trypsin, Pfanstiehl 1:110; and papain, "Caroid" of Amer. Ferment Co.). The enzyme solution and broth supernates (or filtrates) were mixed in equal amounts, and the reactions of the mixtures adjusted with dilute sodium hydroxide or hydrochloric acid. (In the case of papain digestion, thioglycollate was added to a final concentration of 0.1 per cent before adjusting the pH.) Following incubation for two hours at 37° C., the mixtures were readjusted to pH 7.0 and tested in the routine manner for lethal toxicity

TABLE 4

THE EFFECT OF PROTEOLYTIC ENZYMES ON TOXICITY AND HEMOLYTIC ACTIVITY, 2 HOURS AT 37° C.

*Enzyme**	*Conditions of treatment*	*LD_{50} per ml.*	*SDH***
Control, none	Untreated, tested immediately	1000	14
Control, none	pH 3.0	10	1
Control, none	pH 7.0	333	0
Control, none	pH 7.0, thioglycollate added	333	8
Pepsin	pH 3.0	5	0
Trypsin	pH 7.0	5	0
Papain	pH 7.0, thioglycollate added	18	1

* None of the enzyme solutions alone showed hemolytic activity or toxicity for mice when used full strength.

** For dilutions 1:2, 1:4, 1:8, and 1:16.

and hemolysin. Controls were prepared in exactly the same manner, except that saline was substituted for the enzyme solution.

The findings and experimental conditions of a typical experiment are given in Table 4. The lethal toxin is inactivated by both trypsin and papain. The low pH used in pepsin digestion is so destructive in itself that the difference in potency between pepsin-treated and control material is slight. However, this difference appeared consistently in repeated experiments and probably actually represents breakdown by pepsin.

The temperature of digestion used, 37° C., is high enough to bring about considerable inactivation of the hemolysin (see controls in Table 4), so that definite enzyme inactivation is evident only in the case of papain. When the above experiment was carried out at 25° C. and under slightly different conditions of pH, all three enzymes appeared capable of inactivating the hemolysin (Table 5).

SUMMARY AND CONCLUSIONS

A liver digest broth medium containing 2 per cent glucose is found to be satisfactory for the production of lethal toxin and hemolysin by *C. histolyticum*. Toxin production parallels the phase of most active growth of the

organism, reaching a maximum at the end of the logarithmic phase (9-12 hours) following which there is a rapid decline in both hemolytic and toxic activity.

The hemolysin of *C. histolyticum* is an exceedingly unstable substance readily inactivated by heat, acid, or alkali, and surface denaturing forces such as violent agitation. The hemolysin is inactivated within a few days by storing in the presence of air in the cold, and the activity lost can be partially recovered by reduction.

In contrast to the hemolysin, the lethal toxin of *C. histolyticum* is indifferent to oxidation and surface denaturation forces. It is somewhat more

TABLE 5

THE EFFECT OF PROTEOLYTIC ENZYMES ON HEMOLYSIN, 2 HOURS AT 25° C.

Enzyme	*Conditions of treatment*	*SDH**
Control, none	Untreated, tested immediately	13
Control, none	pH 3.1	8
Control, none	pH 8.3	9
Control, none	pH 8.3, thioglycollate added	12
Pepsin	pH 3.1	1
Trypsin	pH 8.3	0
Papain	pH 8.3, thioglycollate added	1

* For dilutions 1:2, 1:4, 1:8, and 1:16.

resistant to heat and shows a different pattern of pH stability. This comparison does not support the suggestion that the two activities are different manifestations of a single substance. These differences might still be explained on the hypothesis that the two activities represent distinct reactive groupings on the same molecule. However, the demonstration that hemolytic activity can be selectively adsorbed from the filtrates, leaving the lethal toxicity unchanged, is direct evidence for their lack of identity.

Both the lethal toxin and hemolysin can be inactivated by proteolytic enzymes, suggesting that both are proteins or protein complexes.

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SERUM VITAMIN A IN NORMAL AND A-DEFICIENT MONKEYS (MACACA MULATTA)*

B. LIONEL TRUSCOTT** AND G. VANWAGENEN

Although a great volume of literature on the effects of vitamin A deficiency exists,[9, 12, 17, 20] relatively few of these studies have dealt with the monkey. Changes in the vaginal epithelium of pregnant monkeys on a vitamin A-deficient diet have been described,[19] and further effects of the diet will be discussed in a coming paper. This report is concerned principally with the vitamin A content of the blood serum in these animals.

The many differences in species prevent a satisfactory comparison of results, especially when the vitamin A values obtained on lower forms are compared with those of primates. Of particular interest and importance, therefore, have been the investigations on human volunteers receiving a diet deficient in this vitamin.[8] The determinations of vitamin A levels of men and women on normal diets,[1, 4, 10, 13] and of women during pregnancy,[1, 2, 3, 5, 6] also provide valuable data as a background for the present study, for the menstrual cycles of women and monkeys have the same modal length.

The lack of quantitative data on vitamin A levels in monkeys on normal and A-deficient diets prompted the present phase of the investigation. Whenever possible, an attempt has also been made to correlate these values with the age, weight, and reproductive cycle of the animal.

MATERIALS AND METHODS

The eighteen adult female monkeys (*Macaca mulatta*) used in this study were members of the Obstetric Colony established in 1931 and were descendants of animals that had been observed for five generations. There was not much precedent for the feeding of these primates, so an inexpensive basic diet of natural foods consisting of potatoes, rice, fresh vegetables and fruits was used and, because repeated breeding was contemplated, adequate protein in the form of fresh milk and eggs, as well as vegetable protein, was furnished. Supplements of all the known vitamins and adequate salt and minerals were either mixed with food or handed individually to the animals. Because there was no specific knowledge of a monkey's requirements, the caloric and vitamin estimates for man were used and adjusted on the basis of weight for this smaller primate.

The A-deficient diet† was based on polished rice and casein. Corn oil, sugar with the usual salts, minerals, vitamins in crystalline form, and liver concentrate free of

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vitamin A were added. The adjustment of older animals to a fixed food mixture was difficult in all cases; some animals refused it and had to be returned to the laboratory diet. Nevertheless, the monkeys used here represent an unusual group in view of the fact that all were over five years old and of known age.

Vitamin A levels were determined by the antimony trichloride method (Kimble, 1939), using a Lumetron photo-electric colorimeter calibrated with the new Vitamin A Reference Standard; 1 cc. of serum was used for each determination, blood being drawn from one of the leg veins.

Results

I. *Normal animals*

a. *Average serum levels.* Average levels of vitamin A in animals of this group showed extremes of 125 I.U. to 165 I.U. per 100 ml. of serum, with

Table 1

Average Body Weights (Grams) and Serum Vitamin A Levels (I.U./100 ml.) in Monkeys on Normal and A-Deficient Diets

	Body weight		*Vitamin A*	
Age (years)	*N*	*A—*	*N*	*A—*
6	7651	5950	149.2	26
7	8935	6890	132.3	37
10	6400	....	129.7	..
11	....	7771	....	43
12	8720	....	159.2	..
13	....	6545	....	82
14	....	9990	....	96

N = normal diet.
A— = A-deficient diet.

a general average of 142.6 I.U. (Table 1). A slightly greater range of values was obtained when all samples were considered individually; the lowest figure determined was 111.5 I.U. as contrasted to the highest of 175.0 I.U. per 100 ml. of serum. In view of the age and weight differences within this group, the above range of values is relatively narrow. This fact receives further emphasis when it is noted that the extreme averages fall 17.6 I.U. below, and 22.4 I.U. above the group average for serum vitamin A in these animals.

b. *Changes during menstrual cycle.* During the first week of the menstrual cycle, the vitamin A content of the serum dropped below the normal average, and rose slightly during the following few days (Fig. 1). Between the 10th and 15th day of the cycle there was an increase in the vitamin level, reaching a peak of 175.0 I.U. per 100 ml. of serum by the 15th day. A relatively high level was also obtained on the 21st day, followed by a sharp decline during subsequent days; by the 24th day, the vitamin A

value was comparable to that on the 5th day, at which time the lowest values were noted (Fig. 1). The serum concentration of the vitamin again reached normal levels by the 27th day.

The attempt to correlate serum values of vitamin A with stages of the menstrual cycle thus provides data of some interest. Outstanding features are the low levels on the 5th and 24th days, and the peak figures obtained around the 15th day. It is apparent that relatively high or low levels were obtained consistently in a given adult female, depending upon the stages of the menstrual cycle at which the blood samples were drawn, and figures obtained from such samples were not representative of the average serum content of the vitamin in this animal or in the group to which it belongs.

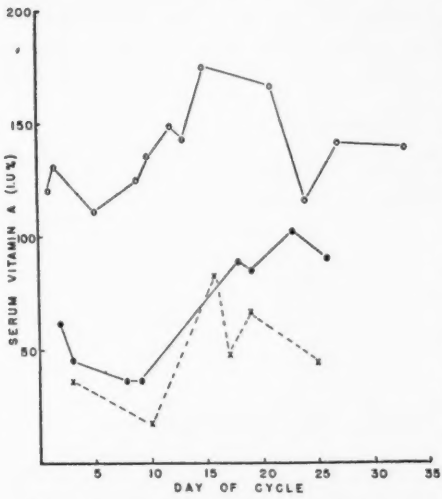

FIG. 1. Serum vitamin A related to the menstrual cycle: average values—Normal: blank circles; A-deficient: black circles; and values obtained following return of the monkeys to a normal laboratory diet: x-x.

c. *Changes with age.* Although the animals comprising this series ranged in age from 5 years and 8 months to 12 years and 9 months, there were no consistent or significant differences in serum concentration of the vitamin between the several age groups (Table 1). A slight but steady tendency toward lower levels was apparent with increasing age of the animal and was most marked between the 6th and 7th year. The smallest value was obtained in a 10-year-old monkey, which exhibited an average of 129.7 I.U. per 100 ml. of serum. The high figure of 159.2 I.U., recorded as the average for a monkey 12 years and 9 months of age, may not be considered as representative because the determinations were performed during the first weeks of pregnancy.

In assembling the above data, special care was taken to avoid interpreting vitamin A differences of the menstrual cycle as being due to the age of the animal. The average values reported were obtained on blood samples at different stages of the cycle in order to prevent this error.

d. *Changes with body weight.* The body weight in normal monkeys showed the greatest increases between the 5th and 7th years; this gain in

weight thus occurred during the period when the concentration of serum vitamin A was declining (Table 1). Body weight by the 10th year was significantly below that recorded in the 7th year. The high weight recorded in a 12-year-old animal cannot be considered as normal for this age because it was pregnant.

II. *Vitamin A-deficient animals*

a. *Average serum levels.* Serum levels of vitamin A in these animals showed wider fluctuation than those noted in control monkeys (Table 1). Extreme averages ranged between 26 I.U. and 96.7 I.U. per 100 ml. of serum, although values of 9 I.U. and zero I.U., respectively, were recorded

TABLE 2

SERUM VITAMIN A (I.U./100 ML.) AND BODY WEIGHT (GRAMS) OF MONKEYS AT VARIOUS INTERVALS AFTER BEING PLACED ON DIET DEFICIENT IN VITAMIN A

Age	*6 months*		*7 months*		*15 months*		*16-18 months*	
(years)	*Vit. A*	*Wt.*	*Vit. A*	*Wt.*	*Vit. A*	*Wt.*	*Vit. A*	*Wt.*
6	26	5950	...	6970	..	....	..	....
10	37	6890	...	6970	..	....	..	....
11	73	7753	57	7790	37	7770	31	8860
13	90	5970	102	6000	81	7240	55	6970
14	88	11310	105	11000	..	7660	..	....
Group average	64	7626	62	8638	59	7610	43	7045

in two animals. The latter figure was excluded in obtaining the group average which was thus 53.3 I.U. (rather than 46.7 I.U.) per 100 ml. of serum. The extreme averages fall 27.3 I.U. below and 43.4 I.U. above the general average for the group. The relative significance of these data is obviously impossible to evaluate without consideration of the age of the animal and the length of exposure to the deficient diet.

b. *Changes during menstrual cycle.* A significant fall in serum vitamin A levels was apparent during the first few days of the menstrual cycle, a more gradual decline was then noted until the 10th day at which time the lowest value was recorded. Subsequent determinations showed a sharp rise in vitamin A content of the serum, with high levels being recorded on the 15th and 23rd days (Fig. 1).

The fluctuations in serum concentration of the vitamin in the deficient group thus showed certain important similarities to those observed in the control group. The low values during the first week, followed by high levels after the 10th day, are probably the most notable features. The sharp decline to normal levels after the 21st day was not seen, although a gradual fall was apparent between the 23rd and 26th days of the cycle. That the changes in vitamin A content of the serum in these animals show so much

similarity to those of the control group is surprising in view of the differences in age and length of exposure to the diet.

Fewer data were obtained on the serum levels of vitamin A during the menstrual cycle after the animals had been taken off the deficient diet, because most of the animals became pregnant. Of some significance, however, is the rapid tendency to approach the normal pattern of the cycle described above (Fig. 1). Low levels were obtained during the first and last weeks, with significant increases noted between the 9th and 16th days of the cycle. A few of these animals exhibited prolonged menstrual cycles immediately after return to the normal diet.

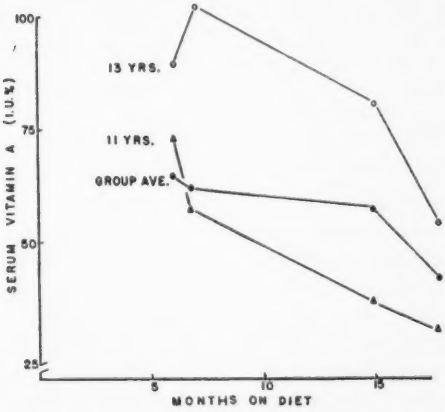

FIG. 2. Average serum levels of vitamin A in monkeys on vitamin A-deficient diet; values for 11- and 13-year-old monkeys are compared with the group average.

c. *Changes with age.* Although all monkeys receiving the vitamin A-deficient diet for 6 to 18 months showed lower serum levels of the vitamin, the depression was less marked in the older animals. The highest levels were obtained from monkeys over 11 years of age, but even these values do not approach the lowest figures obtained in the control group.

d. *Correlation with length of time on diet.* In plotting the serum levels of vitamin A throughout the 18 months on the diet, the averages were obtained from animals of widely different ages. Relatively small changes in concentration of the vitamin were noted during the first 15 months (Fig. 2), after which a more marked decline was noted. Although earlier and more pronounced changes might have been depicted had younger animals been used, it is of interest that even the latter do not exhibit striking effects of the diet until about the 6th to 7th month of the experiment.

e. *Influence of age at start of diet.* The age of the animal when placed on the diet considerably influenced the subsequent changes in serum content of vitamin A. Six months after beginning the experiment, the 6-10-year-old monkeys had markedly low levels of the vitamin as contrasted to the higher, although subnormal, levels in the older animals. Subsequent determinations on the latter group illustrate the effect which age may have on the results. The 11-year-old group exhibited a fall in vitamin levels by

the 7th month, relatively constant, low values being maintained during the following 11 months. Older animals showed a temporary rise, followed by a steady decline in the serum concentration of vitamin A between the 7th and 15th months; between the 15th and the 18th months a greater drop in values was observed. The latter values, however, were significantly above those recorded in the younger group at this time (Table 2).

The early and definite response of the younger group to the deficient diet was duplicated in the changes noted after the diet was discontinued. A progressive and relatively rapid rise in serum content of the vitamin was seen in the first week; and levels approached the lower limits of normal by the fourth week. Contrasted to this is the more irregular and slower response of the older animals, with a tendency to retain the low levels during the first month. Pregnancies unfortunately eliminated too many animals to allow a representative plotting of these changes. The available data, however, indicate that the vitamin A content of the serum remained at subnormal levels for at least three months after the deficient diet had been discontinued.

f. *Pregnancy.* Vitamin A determinations on pregnant monkeys showed that relatively high serum concentrations were reached during the first few months of pregnancy. The one animal in which a fall in the level occurred shortly after conception aborted on the 19th day of pregnancy.

g. *Body weight.* There was a lack of correlation between changes in serum vitamin A levels and the fluctuations in body weight during corresponding periods. A relatively constant body weight was maintained in some animals although marked increases or decreases in vitamin values occurred. This failure to show a parallelism between weight and serum vitamin A is further emphasized when the animals are considered individually. When the group is considered as a whole, however, such a parallelism does exist, thus masking profound variations noted in the individual animals.

The younger monkeys showed a rapid increase in body weight when the deficient diet was discontinued. The older animals, however, gained weight slowly, and some lost weight during the first days or weeks after the experiment was terminated. In these respects, therefore, the correlation of age with changes in body weight is comparable to that observed with changes in serum vitamin A levels.

Discussion

The range of serum vitamin A values in normal monkeys, irrespective of age or body weight, is relatively small in comparison to the range in man;[1, 4, 10, 12] a similar lack of correlation between body weight and vitamin A levels in the blood has also been indicated.[1, 21] A wider range of values, also independent of body weight, was observed in monkeys on a diet deficient in the vitamin.

The above study indicates that certain factors may profoundly affect the vitamin levels in the serum; the first or last weeks of the menstrual cycle may show abnormally low values, while unduly high averages may be obtained if the samples are drawn during the middle third of the cycle.

An additional factor of importance affecting the vitamin A determinations in the deficient animals is the age of the latter at the start of the deficient regime. The younger group exhibited the first significant decrease in serum levels after the 6th to the 7th month, in contrast to the older group which showed little change until after the 15th month on the diet. The return of serum vitamin A to normal values, after the diet had been discontinued, was also more rapid in the younger animals.

The pattern of serum vitamin A levels during the menstrual cycle is of apparent significance to the concept of a relationship existing between this vitamin and the reproductive hormones.[11, 14, 15, 16] Further investigation is essential, however, to determine the true significance of the suggestive data reported.

Summary

1. Serum vitamin A levels in monkeys on the laboratory diet remain relatively constant and comparable, irrespective of weight of the animals; there is a slight tendency toward lower values with increasing age.

2. Vitamin A values in animals on the laboratory diet were low during the first and last weeks of the menstrual cycle, and showed a significant increase during the middle two weeks of the cycle.

3. In the animals on the deficient diet the low vitamin A levels during the first week of the menstrual cycle, followed by higher values during the second week, simulate the pattern of changes noted in the normal animals; the high values were maintained for a longer period, however, in the A-deficient monkeys.

4. After six to seven months on the A-deficient diet young monkeys showed significant decreases in serum content of the vitamin; older animals did not show comparably low values until about fifteen months.

5. With interruption of the deficient diet, serum levels of the vitamin and body weight tended to return more rapidly toward normal in the younger group of monkeys.

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A JACOBEAN VIEW OF OLD AGE*

LEONELL C. STRONG

In 1620, there appeared a book entitled *Resolves, Divine, Morall, and Politicall* by Owen Feltham. During the next eighty years the book was greatly enlarged and went through many editions and reprints. About two hundred topics of widespread interest were discussed, among these being an essay on "The misery of being old and ignorant." A brief résumé of the essay in modern spelling follows:

Since old age is not only a congregation of diseases, but even a disease of itself, and that incurable save by death, the best thing next to a remedy is an abatement of the malady. When infirmities are grown habitual and remediless, all we can do is to give them some respite and a little alleviation that we may be less sensible of the smart and sting they smite us with. The cold Corelian cannot change his clime; but yet with furs and fires he can preserve himself and stove out winter armed with ice and wind. And what thing is there in the fathom of industrious man that can so qualify him against the breaches and decays that age makes in him, as Knowledge, as study and meditation? With this, he can feast at home alone, and in his closet put himself into whatever company that best shall please him, with youth's vigour, age's gravity, beauties' pleasantness, with peace or war, as he likes. It abates the tediousness of decrepit age, and by the divine raptures of contemplation it beguiles the weariness of the pillow and chair. It makes him not unpleasing to the young, reverenced by age, and beloved of all. A grey head with a wise mind enriched by learning is a treasury of grave precepts, experience, and wisdom. He that can read and meditate need not think the evening long or life tedious. It is at all times employment fit for a man.

I doubt not but it was this that in the main produced from Gorgias that memorable answer. Being a hundred and seven years of age, one asked him, "Why he lived so long?" He replied, "Because he yet found nothing in old age to complain of." Certainly, if anything hath power, it is virtue and knowledge that can ransom us from the infirmities and reproaches of age. Without this, an old man is but the lame shadow of that which once he was. They honour him too far that say he is twice a child. There is something in children that carries a becoming prettiness, which is pleasant and of grateful relish, but ignorant old age is the worst picture that Time can draw of man. 'Tis a barren vine in autumn, a leaky vessel ready to drop in pieces at every remove, a map of mental and corporeal weakness, not pleasing to others but a burden to himself. His ignorance and imbecility condemn him to idleness; which to the active soul is more irksome than any employment. What can he do when strength of limbs shall fail and the gust of pleasure which helped him to misspend his youth, through time and languid age shall be blunted and dull? Abroad he cannot stir to partake the variation of the world; nor will others be fond of coming to him when they shall find nothing but a cadaverous man composed of diseases and complaints that for want of knowledge hath not discourse to keep reason company. Like the cuckoo, he may be left to his own moulting in some hollow cell; but since the voice of his spring is gone (which yet was all the note he had to take us with) he is now not listened after: so the bloodless tortoise, in his melancholy hole, lazeth life away. Doubtless, were it for

* From the Department of Anatomy, Yale University School of Medicine.

nothing else, even for this is learning to be highly valued, that it makes a man his own companion without either the charge or the cumber of company. He needs neither be obliged to humour nor engaged to flatter. He may hear his author speak as far as he likes, and leave him when he does please, nor shall he be angry though he be not of his opinion.

Knowledge is the guide of youth, to manhood a companion and to old age a cordial and an antidote. If I die tomorrow, my life to-day will be somewhat the sweeter for Knowledge.

It has always been a matter of interest that the author, Feltham, was only eighteen when the essay on old age was written (he was born in 1602 and died in 1668). Several individuals have pointed out similarities of thought and expression between the "Resolves" and works of Francis Bacon, especially the Essays (*Essayes. Religious meditations. Places of persuasion and dissuasion.* 1597.) Certainly the thought that "it is virtue and knowledge that can ransom us from the infirmities and reproaches of age" is Baconian, and is reminiscent of Bacon's statement to his uncle, Lord Burghley, "I take all knowledge to be my province."

That old age may be relieved by knowledge is a noble concept even though it may be applied to only a small part of an ageing population. We know that the study of old age interested Lord Verulam (Bacon) about the same time since his "Historia vitae et mortis" appeared in 1623.

In fact, the early part of the seventeenth century was rich in its observations on the ageing process. Of this literature the book by Feltham and Lord Verulam's essay are of particular interest to the modern student of gerontology.

WILLIAM THOMAS SALTER
1901-1952

This is our first meeting* since the untimely death, on July 30, 1952, of Dr. William Thomas Salter, Professor of Pharmacology in this School since 1941. It is also the first meeting from which he has been absent in some time. We shall miss his cheerful presence which often went far to dispel the excess gravity which has on occasion crept into the deliberations of this Board.

The record of his work and of his career is familiar to most of us. We were aware that he had been moulded in a certain pattern which was well established before he came, at the age of thirty-nine, to New Haven. Essentially, he was heart and soul a Bostonian who had emigrated south to Yale, and, somewhat to his surprise, had found much that was good here. Educated in solid Bostonian traditions, at Roxbury Latin School, and at Harvard College, where he was elected to Phi Beta Kappa, he graduated in medicine at the Harvard Medical School in 1925. During the subsequent two years he served on the house staff of the Massachusetts General Hospital where he also became a member of the research team, headed by Dr. George R. Minot, which first tested the use of liver extracts in the therapy of pernicious anemia. Subsequently, after a year abroad, which took him to London for research on the thyroid gland, he returned to the Department of Medicine at Harvard in 1929, to serve there for the next twelve years. During this active period of his life he was affiliated in various capacities with a number of Boston hospitals as well as with the Thorndike Memorial Laboratory where he was able to combine biochemical research effectively with clinical medicine—an opportunity, in other words, for true clinical investigation. He also published his first book at this time, *The Endocrine Function of Iodine,* which appeared in 1940.

In 1941 he came to this medical school as Professor of Pharmacology. The eleven years in which he was with us were a short, but busy period to which the expansion of the Department of Pharmacology bears witness. Under his direction it became a University department in the true sense of the word. This was largely due to his own activity directed along special lines of work, on the metabolism of iodine (for which he received, in 1949, a distinguished award from the American Pharmaceutical Association), and in other fields including the metabolism of calcium and phosphate, carcinogenic agents, radio-active isotopes, and drug action.

His other contributions to the welfare of the School were not inconsiderable. Although perhaps as a Bostonian he always claimed to be somewhat

* A minute prepared by Dr. John R. Paul and presented to the Board of Permanent Officers of the Yale University School of Medicine on October 23, 1952.

mystified by the policies governing Yale, he knew what he was best capable of giving, and of this he gave freely in a quiet, unspectacular fashion. His establishment of a series of Pharmacological Seminars was an example of this. Few of his friends may have appreciated that in recent years he turned over the honoraria he received from lectures to a fund, established in memory of his mother, which was used to defray the expenses of out-of-town speakers to these seminars. Another example was his participation, during the war years (of 1942-43), in a project promoted by a small group anxious to see if the School could develop something worthwhile in the field of industrial medicine. To this end he joined in the campaign which raised nearly $100,000, not for his own department, but for the School. In line with this interest he worked for years to establish here a toxocological laboratory which might be worthy of this institution.

We shall also remember Bill Salter for other qualities, notably that of scholarship. He was interested essentially in historical tradition which found good expression in his feeling for what he considered the best use of the *English* language. True to his own medical specialty he was an exponent of the use of "high emotion" in his writing. We also recall his pleasure in the fact that he had been a member, during his years in Boston, of the Classical Club there, where good friends and scholars met throughout the winter to read and discuss Latin and Greek together. We are aware that up to his death he searched for this sort of companionship here.

In November 1944 he suffered a coronary occlusion which had a profound effect upon his subsequent life. He must have been convinced during the last eight years that his days were numbered and once he was launched on the task of preparing an authoritative text on pharmacology, he pushed it forward with feverish activity. It is a source of pride and pleasure to his friends that this book, a monumental work, was completed and off the press in February of this present year. Indeed his last year was a *good* year for him. Those of us who attended his fiftieth birthday party six months before his death will recall with wistful pleasure this occasion when a few of his friends gathered to pay him simple tribute which in retrospect seems all too meagre.

We shall miss him sorely from the School and from these meetings, but the memory of his presence here will not be soon erased. To his wife and to his three daughters, the Board extends its deep sympathy.

EVENTS

WOODWARD LECTURE

October 1, 1952

THE PROBLEMS OF THE ENDOCRINOLOGY OF THE FETUS. By Alfred Jost, Professor of the Faculty of Science, University of Paris.

Hormonal control of the development of the male genital organs in placental animals can be observed by *in utero* surgical castration of rabbit fetuses. When castration is performed on males on fetal day 19, the male organs fail to develop, and differentiation proceeds along female lines; however, castration in the female does not impede sexual development, as evidenced by the continued development of the mullerian derivatives. The return of male characteristics to castrated males can be produced by the administration of testosterone. Bits of fetal testis implanted in the female mesosalpinx produce local atrophy of the female organs and hyperplasia of the male organs. These findings indicate that the fetal testis not only stimulates the growth and development of male organs, but also inhibits the development of female organs.

A relationship between the fetal testis and pituitary is indicated by the finding of inhibition of the development of male structures following hypophysectomy by decapitation between fetal days 19 and 22. Histochemical studies of the fetal pituitary indicate that it is active in producing gonadotropin at this time.

G. McK.

YALE MEDICAL SOCIETY

October 8, 1952

ZONE ELECTROPHORESIS AND THE LIPOPROTEINS IN THE SERUM. By H. G. Kunkel, The Rockefeller Institute for Medical Research, New York, New York.

Electrophoresis in a solid supporting medium, usually filter paper, has been used extensively in the investigation and separation of blood proteins. When filter paper was replaced by potato starch as the supporting medium, better definition of the proteins was obtained. With this medium the mobilities of human sera in health and disease were studied. In a barbital buffer of pH 8.6 at an ionic strength of 0.1 there was a clear separation of α- and β-lipoproteins, the latter showing a high cholesterol-phospholipid ratio. The mobilities characteristic of normal serum were altered in some diseases. In biliary cirrhosis, there was a general tendency for disappearance of the α-lipoproteins; hypercholesterolemic serum showed a high β-lipoprotein content; the serum in nephrosis was characterized by a small β-lipoprotein pattern and a large pattern of intermediary mobility; a pattern of intermediary mobility was also present in hypothyroidism. Alteration of the pH and ionic strength to 7.6 and 0.05 respectively greatly altered the mobility pattern: the well-defined two-component system was replaced by five or six different and overlapping peaks.

J. P. G.

PHYSIOLOGICAL CHEMISTRY SEMINAR

October 28, 1952

THE METABOLISM OF VALINE IN THE RAT. By D. S. Wiggans, Department of Biochemistry, University of Illinois.

It has been shown that valine is probably metabolized by way of isobutyric acid and subsequently as a three-carbon compound. The present investigation attempts to clarify the steps of this reaction. Labeled isobutyric acid was used, in which C^{14} occupied the carboxyl position, while both methyl groups contained C^{13}. Normal male rats, known to be gaining weight, received an injection of the isotopic preparation and were studied for the following: (1) loss of isotope in the urine and feces, and as carbon dioxide, during a twenty-four-hour period; (2) concentration of isotope in certain body constituents at autopsy. The cumulative loss of the two isotopes as carbon dioxide was roughly equal during the collecting period, but it was observed that C^{14} was apparently metabolized much more rapidly than C^{13}, since C^{14} appeared in the expired carbon dioxide in greater amounts during the first few hours. Small concentrations in the urine revealed approximately equal amounts of the two isotopes. At autopsy, however, C^{13} levels were twenty times higher than those of C^{14} in the glycogen and glutamic acid content of the liver. Cystine contained a trace of labeled carbon but tyrosine contained none. It is suggested, therefore, that "the metabolism of isobutyric acid leads to the production of a three-carbon compound derived largely from the isopropyl group which is later converted to glycogen and glutamic acid."

O. L. K.

BOOK REVIEWS

THE PRESENT STATUS OF ANTIBIOTIC THERAPY WITH PARTICULAR REFERENCE TO CHLORAMPHENICOL, AUREOMYCIN, AND TERRAMYCIN. By Francis G. Blake. Springfield, Illinois, Charles C Thomas, 1952. 26 pp. $.90.

This monograph, Publication Number 137 in the "American Lecture Series," is comprised of the Augustus B. Wadsworth Lecture delivered on May 16, 1951 by the late Professor Blake. The spectrum of antimicrobial action of the newer antibiotics is described and their effectiveness compared with penicillin and streptomycin in diseases amenable to treatment with these latter two agents. The difficulty in interpreting therapeutic results is stressed, particularly in diseases which tend to run unpredictable courses, such as Q-Fever, lymphogranuloma venereum, and primary atypical pneumonia. Of great importance in the choice of antibiotic is the establishment of the etiological diagnosis and determination of bacterial sensitivity. The nature and varieties of toxic reactions to antibiotics are only now becoming generally known and caution in their use is advocated. In general, the tone of this four-year interim report is one of optimism tempered with an appeal for wise and judicious use of these important weapons.

R. R. WAGNER

A TRAIL OF RESEARCH IN SULFUR CHEMISTRY AND METABOLISM AND RELATED FIELDS. By Vincent du Vigneaud, Professor of Biochemistry, Cornell University Medical College. Ithaca, New York, Cornell University Press, 1952. xii + 191 pp. 51 figs., 39 tables. $3.25.

It is a pity that what Professor du Vigneaud has done so well in this little volume is not attempted more frequently. Although we have reports and reviews, "recent advances," and the like in abundance, and most of us are duly grateful for such surveys of the current literature, stories of the development of a plan of a research which has extended over a considerable period of years are not at all common. The present volume is thus extremely welcome.

The six chapters comprise material presented in 1950 in the Messenger Lectures at Cornell University on the Evolution of Civilization. In the Preface the author explains how his work on the cystine content of insulin, which was begun in 1925, led to the study of a series of sulfur-containing nitrogenous substances notably djenkolic acid glutathione, and homocystine. This last substance was first prepared by the action of sulfuric acid on methionine, and investigation of it led to the explanation of the formation of cystine from methionine in the animal body through the intermediate substance cystathionine. In all of this work an interesting new organic technique, the reduction of disulfides and the cleavage of benzyl esters and benzyl thioethers with metallic sodium in liquid ammonia, as well as the alkylation of thiol groups in liquid ammonia, played a dominating rôle.

Curiosity regarding the fate of the methyl group in the course of the transformation of methionine to cystathionine led to the surprising observation that a source of methyl groups is essential for rats fed a methionine-deficient diet supplemented with homocystine. This observation suggested that the methyl group can be transferred *in toto* from one compound to

another in the animal body. The full demonstration of the importance of transmethylation and of its relationship to the metabolism of choline, betaine, creatine, sarcosine, and a number of other methylated substances involved research that extended over a number of years. The discussion forms the subject matter of three of the chapters and requires considerably more than one-half of the book. In the last chapter, the author describes briefly the most recent investigations of the complex polypeptide pressor hormones of the posterior pituitary, studies in which modern techniques are applied to the problem of the determination of amino acids and in which the trail of research has led in full circle back to a problem similar to that with which it started, the determination of cystine in insulin.

Taken together, this discussion of the work of the author and his numerous collaborators is an outstanding example of what is best in American biochemistry. It illustrates the application of rigid and relentless chemical logic to the solution of biological problems, some of which were relatively simple but most of which were extremely complex. Inevitably the book will become required reading for all graduate students in biochemistry and it will indeed serve as an encouragement to them, for it shows how the well-planned experimental attack in which nothing is left to chance and no loose ends remain for others to toy with leads in the end to unequivocal advance in knowledge.

H. B. VICKERY

CURRENT THERAPY 1952. Edited by Howard F. Conn. Philadelphia, W. B. Saunders Co., 1952. xxxi + 849 pp. $11.00.

This latest edition of a useful annual publication contains brief, up-to-date, and to-the-point descriptions of the management of most common and many uncommon medical and surgical diseases, each by a recognized authority. It might be expected that small disagreements over some minor therapeutic procedures might appear in a volume with so many contributors and indeed this is the case. However, most major controversial points are treated thoroughly, both sides of the argument being presented in a minimum of space, usually by more than one consultant. Insofar as it is possible to do so, therapy alone is considered, problems of diagnosis or prognosis being outside the scope of this work. This book can be readily recommended as a valuable addition to the reference shelf of the busy practitioner who should keep it within easy reach.

IVAN L. BENNETT

ULTRAVIOLET RADIATION. By Lewis R. Koller. New York, Wiley and Sons, 1952. $6.50.

In general, Dr. Koller has succeeded in accomplishing his purpose which was to collect hand-picked pieces of information for most popular usages from the voluminous literature on ultraviolet radiation. Briefly, the book is divided into the following: (1) sources of ultraviolet radiation, (2) transmission and reflection, (3) applications and effects, and (4) methods of detection.

Arcs, a major source of ultraviolet radiation, are given considerable space, with each general type of arc discussed at some length. One could choose from this discourse the type of arc necessary for a given problem. There is a good comparison of specifications for germicidal lamp.

The chapter on solar radiations is well and interestingly done. This reviewer was surprised to learn how little of the germicidal portion of the ultraviolet spectrum is found in solar radiation.

Ultraviolet transmission through various types of glass, plastics, other solids, and liquids is considered. Solutions for choosing various sections of the ultraviolet spectrum are included. A similar plan has been followed in considering reflecting surfaces. For the most part these sections and the following section on applications and effects are of general interest to scientists who need only a limited amount of information for their respective uses.

D. B. SEELEY

RYPINS' MEDICAL LICENSURE EXAMINATIONS. Edited by Walter L. Bierring. Philadelphia, J. B. Lippincott Co., 1952, Seventh Edition. xvi + 856 pp. $8.00.

This latest edition of Rypins continues to be substantially the same in form and content, i.e., separate summaries of each of the major subjects taught in medical school are presented, followed by a number of questions (without specific answers). The volume offers a compact, yet dependable, review of the medical course. To achieve this, some of the most noted men in their respective fields have been assembled on the author's panel, and considerable editorial care has been taken to prevent reduplication of topics in the separate chapters. In this edition, the chapters on Biochemistry, Microbiology, Preventive Medicine and Public Health have been revised and brought up-to-date.

However, whether a completely dependable review of the medical course can be achieved in 800 pages, even under the finest authorship and editorial care, is really to be doubted. Certainly, this volume has shortcomings which cause it to fall short of that ideal. First, and most glaring, there is no section on Pediatrics. Most of the material that would be considered in such a section is presented in other chapters and, therefore, is properly omitted. But surely discussions of child development and infant feeding would seem indicated. Furthermore, certain chapters requiring revision have not been revised. To offer but two examples, the discussion of pernicious anemia in the chapter on Medicine is somewhat anachronous for a book dated 1952, while omitting a presentation of pre- and postoperative care in the chapter on Surgery is ignoring a phase of that subject in which great advances of recent times have contributed enormously to the steady decline in surgical mortality. Finally, the user of this book should be warned that an apparent attempt to keep the size down, there have been not only the major omissions already noted but countless minor omissions or only brief allusions to many phases of every subject.

Despite all shortcomings, however, the book may be of much use to those wishing very brief reviews of their courses for the purpose of rapid mental organization. Its value is enhanced, I believe, if the user is made fully aware of the weaknesses of the book and if he regards its assimilation not as signaling the attainment of an adequate knowledge of the subject but, at best, as the attainment of an adequate basis for organization of thought and further knowledge.

R. G.

GENERAL BIOLOGY. By Perry D. Strausbaugh and Bernal R. Weimer. New York, John Wiley and Sons, Inc.,1952. 813 pp. $6.00.

An attempt to write a college textbook for general biology poses the problem of striking the happy medium between boring the students who have had advanced courses in biology and those who have had little or no biology. The authors have met this problem adequately, though there are passages where they commit the fault of too obviously talking down to the student. This fault is most apparent in the first chapter where we find, for example, the following sentence: "Dogs, horses, birds, and flies are animals." However, once the authors settle down to presenting the actual subject matter, they do an excellent job of avoiding the twin pitfalls of over-simplification and of making the presentation too involved or too complex.

The sections on respiration, mitosis, meiosis, and heredity are especially noteworthy, as are the diagrams and most of the photographs. The book also contains twenty excellent color plates which are, unfortunately, grouped in one section. I feel that this arrangement somewhat limits their usefulness as opposed to having them distributed throughout the text.

It is odd that with the recent trend toward early sex education, in some cases starting in high school, there is to be found no discussion, or even mention of the mammalian sex act.

The book contains two chapters which present a general view of and introduction to biology, seven chapters on metabolism, four on adjustment to environment, one each on reproduction in plants and animals, one on embryology, two on heredity, ten concerned with taxonomical presentation of plants and animals, three on life: its origin and evolution, and a combined index and glossary.

Some serious faults are the lack of a bibliography, a distracting use of quotations without references, and infrequent footnotes.

One of the most refreshing aspects of this textbook is the authors' attempt to teach the student to apply the scientific method and the information gleaned to everyday living. One way in which they have done this is to "debunk" some of the more extreme advertising claims made by the manufacturers of cigarettes and laxatives.

This book has been written as a teaching aid, and, as such, it facilitates the work of both the student and the instructor. Though there is still room for improvement, this is a superior college textbook for general biology and an improvement over previous editions.

J. M. Q.

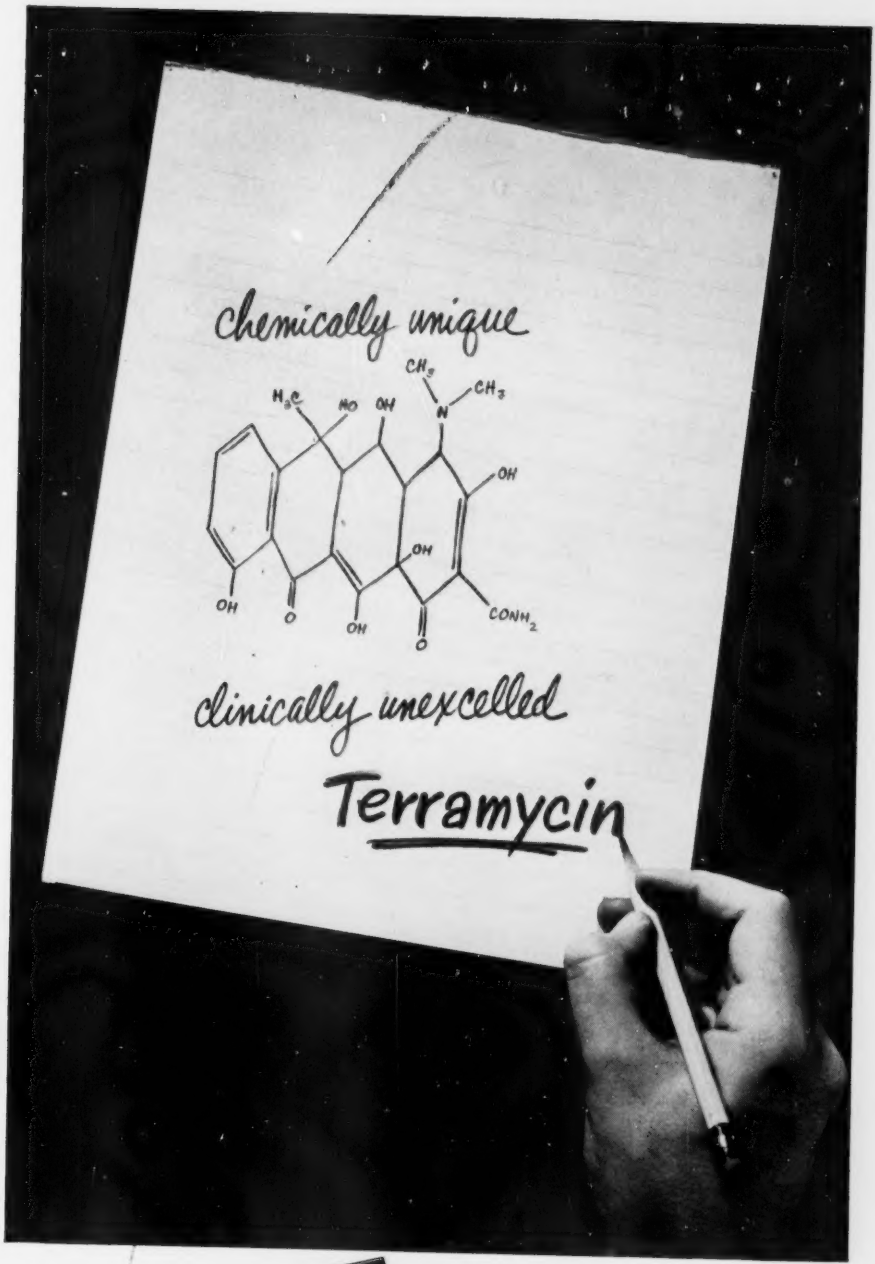
SURGICAL TECHNIQUE. By Stephen Power. Philadelphia, J. B. Lippincott Company, 1952. x + 380 pp. $6.50.

One of the most frustrating and perplexing experiences a student in medicine can have is watching a surgical procedure when he has not had adequate background. Few are the surgeons who give a running commentary on all details, from positioning to knot-tying. Thus, not understanding the why and wherefore of technique, the uninitiated profits little or none by his attendance. One of the merits of this book is the prevention of this confusion by a thorough discussion of basic operating room procedure. Though description of complete operations is beyond the scope of

this volume, most of their component parts are discussed under separate headings such as, "The Approach," "Drains," "Closing the Wound." Special problems receive space in chapters devoted to ducts and fistulae, surgery of children, etc., as well as to broad outlines of some of the major specialties.

This sort of organization cannot fail to give adequate coverage of the field, as well as to make a useful book for quick reference—and such it truly is. The material is presented in a compact and readable fashion, starting out with the most basic essentials and then progressing to the refinements and variations of technique. The volume is worthy of being read by all students of surgery; its coverage of minor surgery and general procedures on the other hand should make it valuable to the general practitioner as well.

N. A. H.



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